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Probing Structure in Invisible Protein States with Anisotropic NMR Chemical Shifts

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Molecular dynamics are often critical for biological function[1] and a detailed understanding of function at the molecular level requires, therefore, knowledge of the average molecular structure *and* how it varies in time. Quantitative information of this sort can be difficult to obtain. For example, many biological processes are predicated on excursions from highly populated and hence observable ground states to states that are invisible to even the most sensitive of biophysical techniques owing to their low population and transient nature. Clearly such excited states will be recalcitrant to study via most methods. However, Carr−Purcell−Meiboom−Gill (CPMG) relaxation dispersion NMR spectroscopy[2−5] has emerged as a powerful technique for studying such excited states, provided that they exchange with the observable ground state on the millisecond time-scale and that they are populated to 0.5% or higher. The CPMG experiment measures line-widths of NMR resonances of the ground state, $R_{2,\mathrm{eff}}$, as a function of the strength of the applied radio frequency field, ν_{CPMG}, to generate relaxation dispersion profiles, $R_{2,\mathrm{eff}}(\nu_{\mathrm{CPMG}})$; the kinetics of the exchange process and the chemical shifts of nuclei in the excited-state are extracted from fits of exchange models to $R_{2,\mathrm{eff}}(\nu_{\mathrm{CPMG}})$.[2]

Chemical shifts are sensitive, site specific probes of structure[6] that can be used to obtain insight into the conformation(s) of the excited state. However, the relation between chemical shifts and structure remains largely empirical and a more quantitative measure is needed to complement the shift information. One possibility is to exploit the powerful structural information inherent in residual anisotropic magnetic interactions that average to zero in an isotropic solution but that can be reintroduced by the addition of alignment media to the sample of interest.[7] One class of anisotropic interaction, the residual dipolar coupling (RDC) between two magnetic dipoles, is routinely used in structural studies of visible ground states of biomolecules.[8,9] These magnetic interactions can also be used to derive detailed structural restraints for invisible excited states by performing CPMG relaxation dispersion experiments on molecules that are fractionally aligned in a magnetic field.[10,11] We have recently shown that it is possible to accurately measure ^{1}H-^{15}N dipolar couplings of invisible states in a pair of applications involving ligand binding to a protein and protein folding.[11]

Other magnetic interactions, which do not average to zero in anisotropic media, can also be measured that provide information complementary to RDCs. For example, in cases where the orientation and magnitude of the chemical-shift tensor are well defined,[12,13] chemical-shift changes upon alignment, ($\delta\varpi$), provide a sensitive measure of molecular structure.[14−16] Such changes are illustrated in Figure 1a for the case of a ^{13}C nucleus that exchanges between ground (A) and excited (B) states with different equilibrium populations and chemical shifts.

Figure 1b illustrates the approach for measuring chemical-shift changes upon alignment in invisible protein states. Here we have chosen an exchanging system that is well described according to

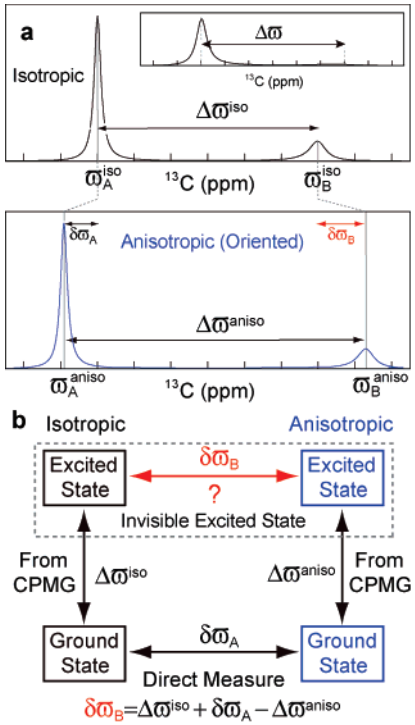

Figure 1. (a) Upon molecular alignment the NMR chemical shifts of the ground (ϖ_A) and excited (ϖ_B) states change, as indicated. In most applications the excited-state is "invisible" (inset). (b) Schematic illustrating the approach used to measure $\delta\varpi_B$, as described in the text.

the scheme,[11] $P + L \rightleftarrows PL$ with forward and reverse rate constants k_{on} and k_{off} ($K_d = 0.55 \pm 0.05\ \mu$M), where P and PL correspond to the free and bound states of the Abp1p SH3 domain and L is a 17 residue peptide. Addition of a small amount of peptide, L, (in the case here $\approx$ 7%) to a solution of ^{15}N, ^{13}C′ (carbonyl) labeled protein, P, (prepared as described in Supporting Information (SI)) produces little change to spectra[11] since only the unbound state of P (corresponding to the ground state) is observed owing to the low concentration and transient nature of the invisible (excited) state, PL (lifetime, $1/k_{off} \approx 4$ ms, as established by relaxation dispersion experiments, see below and SI). Yet high quality ^{13}C′ CPMG relaxation dispersion profiles can be recorded in both isotropic (black, Figure 1b) and anisotropic (blue) phases that reflect the exchange binding process described above. Separate analyses of dispersion profiles recorded in this manner (see SI for details) enable the extraction of site-specific ^{13}C′ chemical-shift differences between P and PL, $\Delta\varpi^{iso} = \varpi_P^{iso} - \varpi_{PL}^{iso}$ and $\Delta\varpi^{aniso} = \varpi_P^{aniso} - \varpi_{PL}^{aniso}$. Carbonyl chemical shifts of the ground state, P, can be measured directly in both isotropic (ϖ_P^{iso}) and oriented (ϖ_P^{aniso}) samples so that $\delta\varpi_A = \varpi_P^{aniso} - \varpi_P^{iso}$ can be calculated; here we have used the same sample for recording dispersion profiles and for measuring

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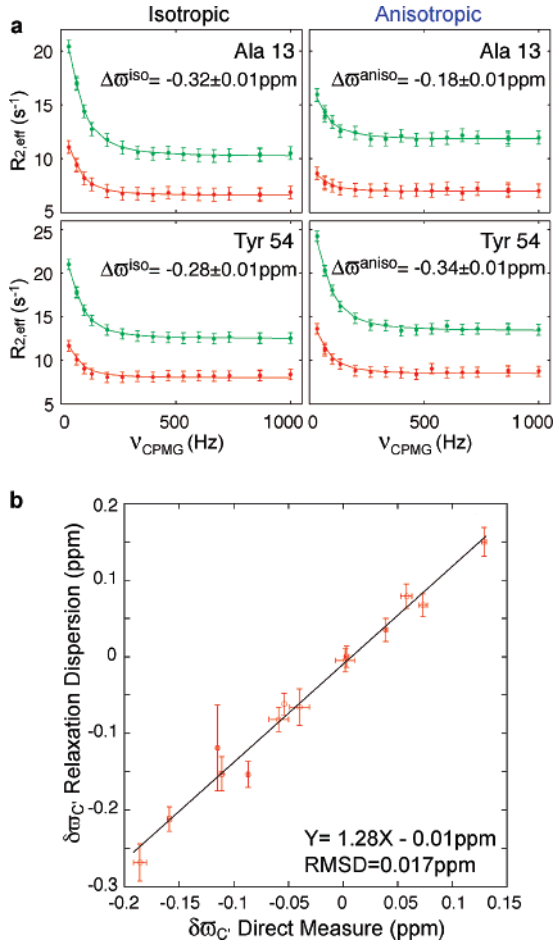

Figure 2. (a) $^{13}C'$ relaxation dispersion profiles measured at 500 (red) and 800 (green) MHz in isotropic and anisotropic phases. Signs for $\Delta\omega$ (not available from dispersion experiments) were obtained from HMQC- and HSQC-type experiments that are described in SI. (b) Correlation between $\delta\varpi_B$ ($=\delta\varpi_{C'}$) values obtained indirectly via relaxation dispersion on a sample with 7% mole fraction ligand and directly via measurement on a sample saturated with ligand. The non-unity value for the slope of the best-fit line through the data reflects the fact that more alignment media was employed in the 7% bound sample relative to the fully bound complex.

the $^{13}C'$ shifts of the ground state since the small amount of added L changes the observed chemical shifts very little from those that would be measured in a solution of only P. Changes in $^{13}C'$ chemical shifts of the invisible state, PL, upon alignment, $\delta\varpi_B = \varpi_{PL}^{aniso} - \varpi_{PL}^{iso}$, can then be calculated from $\Delta\varpi^{iso} + \delta\varpi_A - \Delta\varpi^{aniso}$. The above approach (Figure 1b) holds for any exchange process, not only ligand binding, but by choosing the present example it is possible to *rigorously* validate the methodology. This is accomplished by changing conditions such that the invisible (excited) state becomes the ground state. In general this is of course not possible, but in the case of ligand binding a second sample can be prepared where an excess of ligand is added to P so that PL becomes the observable (ground) state. Both ϖ_{PL}^{aniso} and ϖ_{PL}^{iso} (and hence $\delta\varpi_B$) can be measured directly and subsequently compared to values obtained using the CPMG-based approach described above.

Relaxation dispersion profiles for Ala 13 and Tyr 54, recorded at static magnetic field strengths of 500 (red) and 800 (green) MHz (circles) along with fits of a model of 2-site chemical exchange to the data (solid lines) are shown in Figure 2a. Extracted values of $\Delta\varpi$ are listed as well. The change in sizes of dispersion profiles upon alignment reflect directly the differences between $\Delta\varpi^{aniso}$ and $\Delta\varpi^{iso}$and thus differences between $\delta\varpi_A$ and $\delta\varpi_B$ that arise because of changes in structure and/or alignment between ground and excited states. Despite the fact that such differences are small, 140 and 60 ppb for Ala 13 and Tyr 54, respectively, it is nevertheless possible to quantify them accurately. This is shown in Figure 2b where a linear correlation plot is presented between $\delta\varpi_B$ values (i) measured indirectly using CPMG relaxation dispersion in the case that PL is the invisible (excited) state and (ii) measured directly when PL is the visible (ground) state. The agreement is impressive and at this level of accuracy the $\delta\varpi_B$ values measured via CPMG-based methods are powerful restraints for structural studies.[16] Further, as this method is insensitive to ^1H spin-flips which can adversely affect ^1H-^{15}N RDC measurements of invisible states,[11] it can provide structural restraints for residues with short selective ^1H T_1s where excited state RDC values are compromised.

In summary, a method is presented for quantifying structure in invisible, excited protein states that exploits anisotropic chemical shifts. Chemical-shift changes upon alignment[15,16] and residual dipolar couplings[7,9] have been shown to be very useful for NMR structural studies of "visible" ground states of proteins. It is anticipated that they will also prove extremely valuable in determining structures of invisible, excited protein states that are recalcitrant to analysis using conventional tools of structural biology.

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Supporting Information Available: Experimental details; pulse sequence used to measure $^{13}C'$ chemical shift differences along with a table of the differences. This material is available free of charge via the Internet at http://pubs.acs.org.

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