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Determination of Leu Side-Chain Conformations in Excited Protein States by NMR Relaxation Dispersion

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Protein function is often predicated on the interconversion between highly populated, ground state conformers and low-populated, excited state structures. These excited states are 'invisible' to most of the traditional tools of structural biology but they can be characterized by the Carr−Purcell−Meiboom−Gill (CPMG) relaxation dispersion NMR experiment,[1] so long as their lifetimes are between 0.5 and 10 ms and their population (p_E) exceeds 0.5%. The kinetics and thermodynamics of a variety of processes involving excited states, including enzyme catalysis,[2,3] ligand binding,[4,5] and protein folding,[6] have been derived from CPMG experiments. These experiments also provide chemical shifts and orientations of bond vectors of the excited state, which form the basis for structure determination of these invisible conformers.[7,8] Very recently it has been shown that for small proteins with $p_E > 2-3\%$ and exchange rates on the order of several hundred/s it is possible to extract methyl-containing side-chain dynamics parameters of the excited state,[21] relating directly to the conformational entropy of the side-chain.[9] Herein, we extend the methodology by providing a very sensitive approach for determining the relative rotamer populations of Leu side-chains in low populated, invisible states.

Leu side-chain χ_2 rotamer populations in ground states of proteins have previously been studied through measurement of three-bond $^{13}CH_3-C^{\alpha}$ scalar couplings;[10] however it has recently been shown that the chemical shifts of $C^{\delta1}$ and $C^{\delta2}$ nuclei provide a very fast and sensitive measure of Leu side-chain conformation as well[11,12] via

$$\Delta\delta(^{13}C^{\delta}) = \delta(^{13}C^{\delta1}) - \delta(^{13}C^{\delta2}) \approx -5 \text{ ppm} + 10 \text{ ppm} \times p_{trans} \quad (1)$$

where p_{trans} is the fractional population of the trans conformation[12] (Figure 1). Central to the interpretation of Leu methyl chemical shifts in this way is that data from a set of high resolution X-ray structures establish that the Leu χ_2 dihedral angle samples only two conformations, trans (t) and gauche+ (g+),[13,14] with $\langle p_{trans}\rangle \approx 0.67$ and $\langle p_{gauche+}\rangle \approx 0.33$[14] (Figure 1), where the brackets denote the average over all structures. These values for p_{trans}, $p_{gauche+}$ are in good agreement with what is obtained for small branched alkane fragments.[15] Moreover, for methyl groups with *B* factors less than 20 Å^2, the bimodal distribution of the χ_2 angle is centered at 174.5° ± 8.4° (trans) and 64.0° ± 7.7° (gauche+).[13,14] The two narrow distributions of χ_2 angles within both the trans and gauche+ conformers support the argument that motion about the Leu χ_2 dihedral angle can be well described by jumps between these two rotameric states. In addition, it is almost always the case that

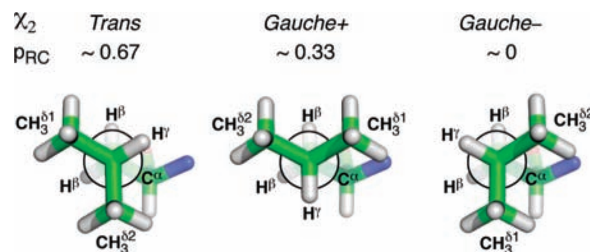

Figure 1. Three Leu χ_2 side-chain conformations with approximate populations obtained from a database of high resolution crystal structures[14] and from studies of small organic compounds that effectively model the random coil protein environment, p_{RC}.

(χ_2, χ_1) = (g+,t) or (t,g−) so that measurement of $\Delta\delta(^{13}C^{\delta})$ allows full characterization of both (χ_1,χ_2) rotameric states.[11,13,14]

In the studies reported here $^{13}C^{\delta1}/^{13}C^{\delta2}$ chemical shift differences between ground and excited conformers, $|\Delta\varpi|$, are readily measured from very sensitive methyl CPMG relaxation dispersion experiments[16] with the signs of $\Delta\varpi$ obtained from peak positions in HSQC/HMQC data sets recorded at several static magnetic fields.[17] Values of excited state chemical shifts, $\delta^E(^{13}C^{\delta1})$ and $\delta^E(^{13}C^{\delta2})$, generated in this manner are converted to p_{trans} via eq 1.

In what follows we consider mutants of the SH3 domain from the Fyn tyrosine kinase, which fold via a low populated intermediate, F⇌I⇌U, where F, I, and U are the folded, intermediate, and unfolded states, respectively. At room temperature the G48M mutant of the highly deuterated Fyn SH3 domain exchanges between F and U with only a negligible population of I (≈4% U and <1% I).[18] By contrast, the A39V/N53P/V55L mutant exchanges primarily between F and I with little accumulation of U (≈2% I and 0.1% U).[19] Thus, the conformations of Leu side-chains of the invisible I and U states can be obtained separately by recording methyl dispersion experiments on each mutant.

Figure 2 shows representative relaxation dispersion profiles obtained from studies of the G48M Fyn SH3 domain. Because the excited state corresponds to the unfolded form of the protein for which p_{trans} values ≈ 0.67 are predicted,[12] the values of p_{trans} obtained in this analysis can be used to validate the methodology. Analysis of the dispersion data to a model of two-site chemical exchange (see Supporting Information (SI)) gives $\delta^E(^{13}C^{\delta1})$ = 25.29 ppm, $\delta^E(^{13}C^{\delta2})$ = 24.70 ppm for Leu18 (Figure 2) from which p_{trans} = 0.69 in the excited state is calculated.

Figure 3a shows p_{trans} values derived for the G48M Fyn SH3 domain in its folded (blue) and unfolded (red) states. All Leu χ_2 values are primarily in the trans conformation in the F state, in agreement with the X-ray structure of this domain.[20] Values of p_{trans} range between 59% and 84% (for F), indicating different levels of dynamics, consistent with measurements of methyl axis order

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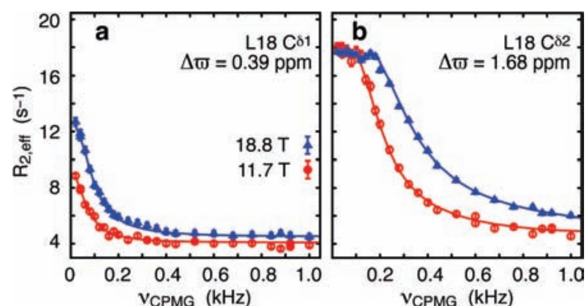

Figure 2. Relaxation dispersion profiles of (a) Leu18 $C^{\delta 1}$ and (b) Leu18 $C^{\delta 2}$ of G48M Fyn SH3 recorded at magnetic field strengths of 18.8 T (blue) and 11.7 T (red). The population of the unfolded state is $p_U = 4.3\% \pm 0.5\%$, and the exchange rate constant is $k_{ex} = 306\ s^{-1} \pm 40\ s^{-1}$. The chemical shift difference $\Delta\varpi = \varpi_F - \varpi_U$, where ϖ_U (ϖ_F) is the chemical shift of $^{13}C^{\delta}$ in the unfolded (folded) state (see SI).

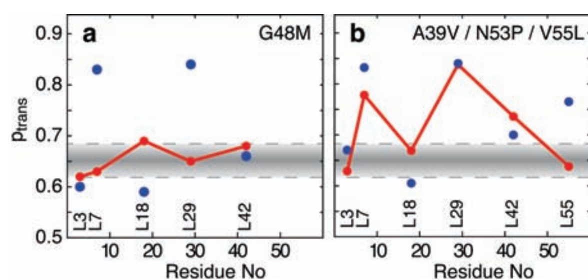

Figure 3. Population of the trans χ_2 conformation, p_{trans}, for (a) G48M Fyn SH3 and (b) A39V/N53P/V55L Fyn SH3 ground (blue) and excited (red) states. Proteins produced as described in SI. The gray shaded area encompasses the p_{trans} values (*average* ± *rmsd*) of the unfolded state of G48M Fyn SH3.

parameters showing a range of side-chain mobility throughout the folded protein.[21] In contrast, p_{trans} values for Leu residues of the U state, derived from CPMG relaxation dispersion data, are centered at 65% (65.3 ± 2.9%), as expected, in good agreement with our previous study of the motional properties of Ile, Leu, Val residues in the U state of the G48M Fyn SH3 domain, where all Leu side-chains were shown to be highly dynamic.[21]

The A39V/N53P/V55L Fyn SH3 domain exchanges between F and I[19] with a rate constant of $k_{ex} \approx 750\ s^{-1}$, 20 °C. The p_{trans} values for the F (blue) and I (red) exchanging states are shown in Figure 3b. It is clear that Leu side-chain dynamics in the folding intermediate are different than that in the unfolded state. In the former a nonhomogeneous distribution of p_{trans} values are observed, with individual p_{trans} distinct from 0.66, consistent with previous findings of significant hydrophobic packing in the intermediate.[19]

In summary, we have shown that conformational sampling about χ_2 of Leu side-chains in excited protein states can be determined from CPMG relaxation dispersion experiments. The experiments are very sensitive, with chemical shifts obtained from fits of CPMG relaxation dispersions accurate to ~0.1 ppm,[22] and it is estimated that the methodology will be applicable to systems with molecular weights at least as large as 30−40 kDa. In the case of the protein folding reactions considered here, a comparison of p_{trans} values of ground and excited states provides a powerful probe of the extent of formation of native-like structure in the excited state in the vicinity of the Leu probes. Moreover, changes in p_{trans} between pairs of states can be used to provide estimates of changes in conformational entropy. Unlike entropy values that are calculated from order parameters that are sensitive to motions on the ps−ns time scale,[9] the approach described here reports on dynamics extending from the ps to the ms regime. The present experiment adds to a growing list of methods for studying the structure and dynamics of excited protein states that promises to bring into focus these elusive, yet biochemically important conformers.

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Supporting Information Available: Details of protein production and NMR data analysis as well as tables of chemical shifts of ground and excited states. This material is available free of charge via the Internet at http://pubs.acs.org.

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