Protein Dynamics

DOI: 10.1002/ange.201209118

Probing Slow Chemical Exchange at Carbonyl Sites in Proteins by Chemical Exchange Saturation Transfer NMR Spectroscopy**

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Protein molecules are constantly interconverting between distinct conformers[1,2] with potentially very different populations, lifetimes, three-dimensional structures, and consequently functions. Determining the kinetics and thermodynamics of these exchange processes along with the structures of the exchanging states is thus necessary for obtaining a detailed understanding of how these molecules function. Traditional crystallography or NMR spectroscopy is used to generate the structure of the major "ground" (G) state, but neither methodologies can be routinely applied to obtain conformations of sparsely populated and transiently formed minor "excited" (E) states. Over the past decade, however, a suite of NMR experiments has been developed for studies of "invisible" protein conformers.[3–5] Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion experiments have emerged for obtaining protein backbone ^{1}H, ^{15}N, and ^{13}C chemical shifts so long as the states involved are populated to greater than 0.5% with lifetimes in the 0.5–5 ms range.[4,5] These chemical shifts then form the basis for structure determination. Conformers populated to less than 10% with lifetimes on the order of 5–50 ms have been particularly recalcitrant to study using CPMG techniques, but recently the magnetic resonance imaging (MRI) chemical exchange saturation transfer (CEST) experiment[6,7] has been adapted[8] to study exchanging protein systems in this regime, focusing on backbone amide ^{15}N[8,9] and methyl side-chain ^{13}C spins.[10] Backbone ^{13}CO chemical shifts would be a useful additional probe because of their sensitivity to secondary structure. Here we present a ^{13}CO-based CEST experiment and use it to study the folding transition of the four-helix bundle FF domain.[11,12]

Since the pioneering work of Forsen and Hoffman,[13] double resonance experiments have been used to characterize the kinetics of exchanging systems and to assign nuclei in the interconverting states. For simplicity, consider a spin in a molecule that exchanges between two conformations, $G \underset{k_{EG}}{\overset{k_{GE}}{\rightleftharpoons}} E$, with fractional populations $p_G \gg p_E$, and where $|\Delta\omega_{GE}^{EG}| \gg k_{ex} = k_{GE} + k_{EG}$ with $\Delta\omega_{GE} = \omega_E - \omega_G$, and ω_j are the resonance frequencies ($rad\,s^{-1}$) in state $j \in \{G,E\}$. When a weak B_1 field is applied near one of the two resonance frequencies, the intensity of the second resonance is affected because molecules are continuously exchanging between the two states. Thus irradiation at the resonance position of the spin in state E leads to loss of magnetization in state G so that ω_E can be determined, even when the excited state is invisible in traditional NMR spectra,[6,7] as in the example considered here.

The pulse sequence for the ^{13}CO CEST experiment (Figure 1) is based on the triple resonance HNCO scheme,[14] with magnetization transfer summarized in Equation (1),

$$^{1}HN \xrightarrow{^{1}J_{HN}} {}^{15}N \xrightarrow{^{1}J_{NCO}} {}^{13}CO(CEST) \xrightarrow{^{1}J_{NCO}} {}^{15}N(t_1) \xrightarrow{^{1}J_{HN}} {}^{1}HN(t_2) \quad (1)$$

where the involved scalar couplings are indicated. Of note, a CPMG-INEPT element[15] is used to transfer magnetization from ^{15}N to ^{13}CO to reduce the effects of chemical exchange that lead to magnetization losses. In addition a weak ^{13}CO B_1 field at a desired offset is applied between points a and b of Figure 1 (duration T_{EX}). By analyzing peak intensities from the major state as a function of B_1 offset, exchange parameters and ω_E values can be obtained,[9] as illustrated below (see also the Supporting Information).

The ^{13}CO CEST experiment of Figure 1 has been used to study the folding of the A39G mutant FF domain[9,11,12] of human HYPA/FBP11, 1 °C. We have shown previously in CPMG studies of the wild-type (WT) FF domain that folding proceeds via a compact on pathway intermediate.[11] It is of interest to establish whether the A39G mutant also folds via an intermediate and if so whether its structure is changed by the mutation. The effects of mutations on sparsely populated protein states that are potentially more "delicate" are much less well understood than for their ground-state counterparts, where a wealth of data has accumulated over decades of experiments.[16] Figure 2A shows the ^{1}H–^{15}N spectrum of A39G FF with peaks observed only for the ground-state correlation (labeled according to residue number). Nevertheless the positions of the corresponding excited-state correlations can be established by CEST methodology, illustrated for the ^{13}CO-based experiment in Figure 2B. By recording a series of 2D ^{1}H–^{15}N (HNCO projection) maps as a function of the position of the ^{13}CO B_1 field and plotting the intensity of the correlation from Ala 53, the CEST profile of Figure 2B is obtained, with the large and small dips centered at the ^{13}CO resonance frequencies of Leu 52 in the ground

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[**] We would like to thank Dr. Guillaume Bouvignies for providing the ChemEX program used in data analysis and Dr. Eriks Kupce for valuable discussions. L.E.K. holds a Canada Research Chair in Biochemistry. The work was supported by grants from the CIHR and NSERC Canada.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201209118.

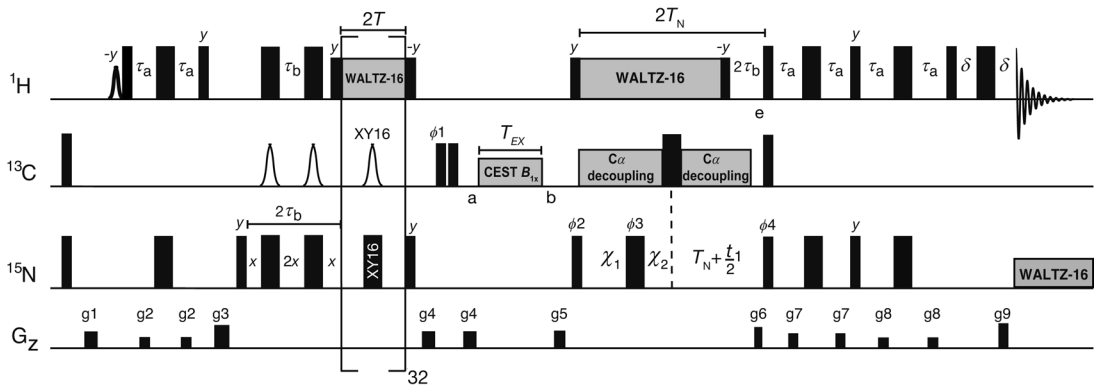

Figure 1. Pulse scheme of the ^{13}CO CEST experiment to study conformational dynamics at backbone carbonyl sites in [U-^{15}N,^{13}C] proteins. The carbonyl CEST profile is obtained by applying a weak ^{13}C B_1 field during a relaxation period, T_{EX}. Narrow and wide black rectangles denote pulses with 90° and 180° flip angles, respectively. The height of each pulse relates to the power at which it is applied, with the tallest rectangles denoting pulses at maximum power. All pulses are applied along the *x* axis unless indicated otherwise. More details are provided in the Supporting Information.

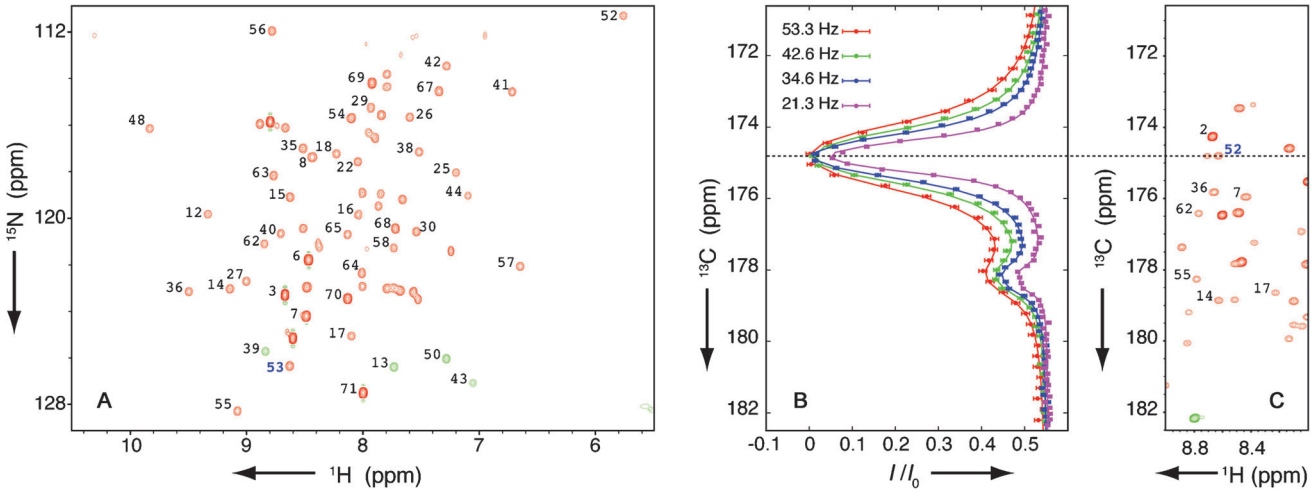

Figure 2. The ^{13}CO CEST experiment "visualizes" the invisible E state of A39G FF. A) Reference ^{1}H–^{15}N plane recorded with the ^{13}CO CEST scheme of Figure 1; T_{EX} = 0, 1 °C. B) G and E state peaks are clearly visible in the CEST profiles for the carbonyl carbon of Leu 52. Here the intensity of the Ala 53 peak is quantified (blue in A) for T_{EX} = 300 ms (I) as a function of the offset of the CEST B_1 field and normalized relative to the intensity for T_{EX} = 0 (I_o). Data sets with four different B_1 fields are measured. C) 2D ^{1}H–^{13}CO correlation map recorded using an HNCO experiment confirms that the G state dip in intensity profile (B) occurs at the same frequency as the ^{13}CO of Leu 52 (blue in C). Peaks are labeled according to the carbonyl shift in C. Correlations in green are aliased.

(ϖ_G, ppm; Figure 2C) and excited states (ϖ_E), respectively. Similar exchange parameters (k_{ex} and p_E) are obtained from independent global fits of ^{13}CO ($68.5 \pm 6\ s^{-1}$, $1.35 \pm 0.09\%$, reduced $\chi^2 = 1.1$) CEST data recorded at four different B_1 fields and ^{15}N ($60.4 \pm 3.5\ s^{-1}$, $1.43 \pm 0.05\%$, reduced $\chi^2 = 1$) CEST data measured at a pair of B_1 fields assuming a two-state model of exchange (Figure S1 in the Supporting Information). The ^{15}N and ^{13}CO CEST data could also be well-fit together ($63.2 \pm 2.6\ s^{-1}$, $1.38 \pm 0.04\%$, reduced $\chi^2 = 1.05$). Parameters were not as well defined when ^{13}CO CEST data at two B_1 field strengths were used ($76.7 \pm 11\ s^{-1}$, $1.24 \pm 0.12\%$; Figure S2), probably because the ^{13}CO-CEST experiment is about 1.5-fold less sensitive than its ^{15}N counterpart because of the extra magnetization transfer steps in HNCO relative to HSQC schemes. An additional source of sensitivity loss stems from the fact that we have used a [U-^{15}N,^{13}C] labeled sample so that ^{13}CO-CEST dips are broadened by $^{1}J_{CO\text{-}C\alpha}$ (51 Hz) and $^{1}J_{N\text{-}CO}$ (15 Hz) couplings. Splittings from these couplings are not resolved for the B_1 fields we have chosen (≥ 20 Hz), although for smaller B_1 values the $^{1}J_{CO\text{-}C\alpha}$ couplings can be seen and we have not succeeded in decoupling them without introducing large modulation sidebands. We have taken the $^{1}J_{CO\text{-}C\alpha}$ couplings into account in fits of data (see the Supporting Information). Although samples that are [U-^{15}N], selectively ^{13}C labeled at the CO position would avoid this problem, current approaches for specific labeling at ^{13}CO sites lead to low levels of enrichment for some amino acids that would nullify any benefits.[17] Of note, the excited-state ^{15}N chemical shifts obtained from measurements on the [U-^{15}N,^{13}C] sample are very similar (root-mean-square deviation, RMSD, of 0.3 ppm) to those reported previously[9] using a [U-^{15}N] labeled A39G FF sample (Figure S2) and the exchange parameters are similar as well ($60.4 \pm 3.5\ s^{-1}$, $1.43 \pm 0.05\%$ versus $51.6 \pm 1\ s^{-1}$, $1.65 \pm 0.02\%$;

differences likely reflect small changes in sample conditions or additional couplings that are not accounted for in the analysis of profiles recorded using the [U-^{15}N,^{13}C] sample).

The amide nitrogen and carbonyl chemical shift differences between the ground and excited states as measured by CEST, $\Delta\varpi_{CEST}$ ($\varpi_E-\varpi_G$) values are consistent with those predicted for an unfolding transition,[18] $\Delta\varpi_{FU}$ (Figure 3 A,B). However, a small systematic deviation is observed with

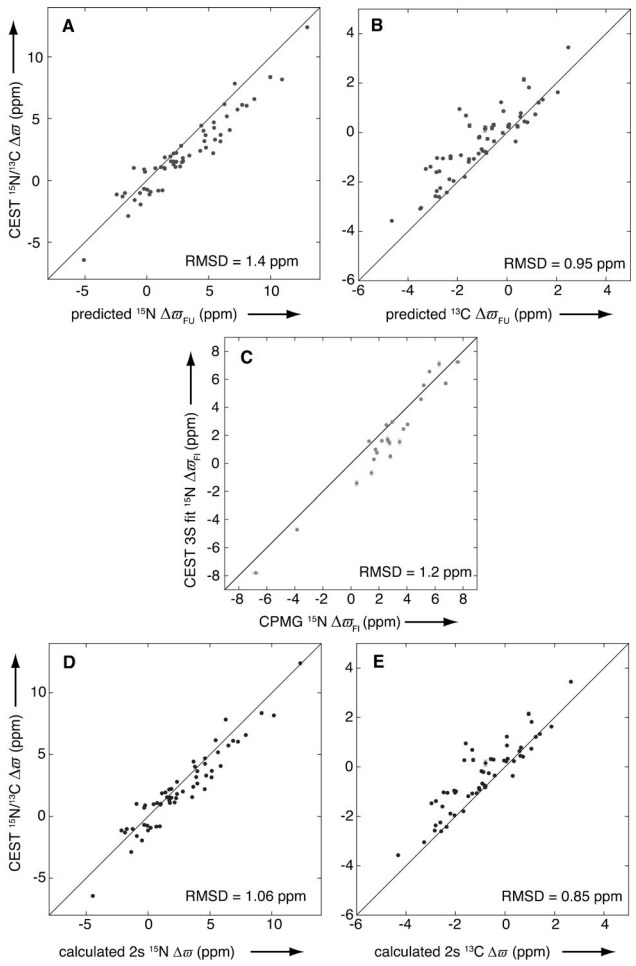

Figure 3. A39G FF folds via an intermediate state, as detected by ^{15}N- and ^{13}CO CEST experiments. Comparison of predicted ^{15}N (A) and ^{13}CO (B) $\Delta\varpi$ values assuming an unfolding transition ($\Delta\varpi_{FU}$, *x* axis) with those obtained via CEST (CEST $\Delta\varpi$, *y* axis) measured on a [U-^{15}N,^{13}C] sample of A39G FF, 1 °C. A two-site exchange model has been assumed. C) Correlation between ϖ_I values from fits of CEST profiles to a 3-site exchange model (FIU, A39G FF, *y* axis) and intermediate chemical shifts from analysis of CPMG data recorded on the WT FF domain (*x* axis). D,E) The systematic deviations in (A,B) are reduced by taking into account the *I* state chemical shifts, as described in the text.

$\Delta\varpi_{CEST} < \Delta\varpi_{FU}$ for ^{15}N, whereas the opposite trend holds for ^{13}CO. The simplest interpretation is that the exchange mechanism is more complex than two state; that is, the folding reaction involves an intermediate, as observed for the WT protein.[11] That such an intermediate is present was already suggested in a previous ^{15}N CEST study of A39G FF where many of the excited-state resonances had large linewidths, that arise because of a rapid exchange between the unfolded (U) state and an additional conformer.[9] However, further analyses were not carried out at that time. The observed discrepancies between $\Delta\varpi_{CEST}$ and $\Delta\varpi_{FU}$ values can be rationalized assuming that A39G FF folds as the WT protein for which the mechanism F⇌I⇌U (FIU; F = native, I = intermediate) has been established,[11] where $k_{ex,FI} \ll k_{ex,IU}$ and $p_F \gg p_U, p_I$. Assuming that the I/U exchange is on the fast/intermediate time scale, the U state peaks will be shifted towards the I state. Because $\Delta\varpi_{CEST} < \Delta\varpi_{FU}$ for many ^{15}N sites it follows that $\varpi_F < \varpi_I < \varpi_U$, whereas the opposite situation for backbone carbonyl groups implies that in the case of ^{13}CO shifts $\varpi_U < \varpi_I < \varpi_F$. Since ^{15}N chemical shifts are smaller than random coil values for helical structure, while ^{13}CO shifts are larger[19] it follows that the I state for A39G FF contains a significant amount of helical structure, as would be predicted on the basis of the atomic resolution model of the WT FF folding intermediate.[11,20]

To elucidate the structural properties of A39G more rigorously we have fit ^{15}N CEST data recorded on a [U-^{15}N] sample to the FIU three state (3S) model above to obtain $p_I = 0.37 \pm 0.02\%$, $p_U = 1.32 \pm 0.02\%$, $k_{ex,FI} = 277 \pm 10\ s^{-1}$, and $k_{ex,IU} = 2318 \pm 100\ s^{-1}$ (see the data analysis in the Supporting Information). Importantly ϖ_I values obtained from the 3S fit of the CEST data are in good agreement with the corresponding intermediate state shifts from WT FF (Figure 3 C) determined using CPMG methods,[11] strongly supporting the three state model, validating the extracted exchange parameters and, significantly, establishing that the A39G and WT FF domains fold via a common intermediate state. The 3S fitted kinetic parameters and populations, along with the observed ϖ_F, predicted ϖ_U, and CPMG WT FF derived ϖ_I values were used to calculate the expected ^{15}N amide and ^{13}CO CEST $\Delta\varpi$ values that would be obtained from fits to a two-state (2S) exchange model. The calculated $\Delta\varpi$ values are in better agreement (RMSD 1.06 versus 1.4 ppm for ^{15}N and 0.85 versus 0.95 ppm for ^{13}CO) than predicted values assuming a 2S exchange between F and U (Figure 3 D,E).

In summary we have presented an experiment to study slow timescale dynamics at backbone ^{13}CO sites in proteins. Extracted $\Delta\varpi$ values from ^{13}CO- and ^{15}N-CEST data sets recorded on A39G FF are consistent with the presence of a folding intermediate, as observed previously in CPMG studies of the WT FF domain. A comparison of ϖ_I values establishes that the I states are similar for both A39G and WT FF so that the mutation does not significantly change the structure of I. Further analyses of the sort presented here will provide insight into the plasticity of excited protein states that can be compared with a wealth of mutagenesis data that has been measured for ground-state protein conformations.

Experimental Section

A 1.5 mM [U-^{15}N,^{13}C] A39G FF sample, dissolved in 50 mM sodium acetate, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM NaN_3 10% D_2O buffer (pH 5.7), was used for measurement of CEST profiles. The protein was expressed and purified as described earlier.[9,12] All NMR experiments were per-

formed at 1 °C on an 11.7 T (500 MHz ^{1}H) Varian Inova spectrometer equipped with a room-temperature triple resonance probe. See the Supporting Information for more details.

Received: November 14, 2012
Published online: February 28, 2013

Keywords: excited states · NMR spectroscopy · protein dynamics · protein folding · structure elucidation

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