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Measuring $^{1}H^{N}$ Temperature Coefficients in Invisible Protein States by Relaxation Dispersion NMR Spectroscopy

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Abstract

A method based on the Carr-Purcell-Meiboom-Gill relaxation dispersion experiment is presented for measuring the temperature coefficients of amide proton chemical shifts of low populated 'invisible' protein states that exchange with a 'visible' ground state on the millisecond time-scale. The utility of the approach is demonstrated with an application to an I58D mutant of the Pfl6 Cro protein that undergoes exchange between the native, folded state and a cold denatured, unfolded conformational ensemble that is populated at a level of 6% at 2.5°C. A wide distribution of amide temperature coefficients is measured for the unfolded state. The distribution is centered about –5.6 ppb/K, consistent with an absence of intra-molecular hydrogen bonds, on average. However, the large range of values (-standard deviation of 2.1 ppb/K) strongly supports the notion that the unfolded state of the protein is not a true random coil polypeptide chain.

Keywords

CPMG Relaxation Dispersion; Temperature Coefficients; Amide Protons; Cold Denaturation

Proteins are not rigid molecules. Often they undergo significant conformational fluctuations leading to transiently populated sub-states that may be critically important for function (Boehr et al., 2006; Henzler-Wildman et al., 2007; Ishima et al., 1999; Karplus & Kuriyan, 2005). These dynamics, involving the inter-conversion of a ground (G), highly populated state and at least one low-populated, excited state (E) are invisible to most standard structural biology techniques (Palmer et al., 2001). Yet so long as the exchange processes involve states with lifetimes on the order of 0.5–5 ms, a highly populated ground state and excited state fractional populations in excess of approximately 0.5% they can be studied in some detail (Korzhnev & Kay, 2008; Palmer et al., 2001) using Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion NMR spectroscopy (Carr & Purcell, 1954; Meiboom & Gill, 1958). These experiments quantify transverse relaxation rates of NMR probes attached to the visible ground state conformer as a function of the spacing between successive refocusing pulses applied as a pulse train. Using suitably labeled samples and recently developed pulse schemes it is possible to measure ^{15}N, $^{13}C^{\alpha}$, $^{13}C^{\beta}$, ^{13}CO, $^{1}H^{N}$, $^{1}H^{\alpha}$ chemical shifts (Hansen et al., 2008; Ishima et al., 2004; Loria et al., 1999; Lundstrom et al., 2008; Lundstrom et al., 2009; Lundstrom & Kay, 2009; Tollinger et al., 2001), ^{1}H and ^{13}C

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Supporting Information

Details of protein production, NMR measurements and data analysis, 1 figure showing fits of dispersion/chemical shift profiles with temperature (for Q27 and R49) and a table of temperature coefficients for the ground and excited states of Pfl6 I58D is provided.

methyl shifts (Baldwin et al., 2010; Lundstrom et al., 2007) as well as anisotropic interactions such as residual dipolar couplings (Vallurupalli et al., 2007) and chemical shift anisotropies (Vallurupalli et al., 2008a) of excited state conformers that together form the basis for structure determination of these excited states (Korzhnev et al., 2010; Vallurupalli et al., 2008b).

One parameter that has not being exploited to date in studies of excited states is the amide proton temperature coefficient, $\Delta\tilde{\delta}_{HN}/\Delta T$, that is sensitive to hydrogen bonding in proteins (Baxter & Williamson, 1997; Dyson et al., 1988). Studies of folded proteins for which high resolution crystal structures are available establish that in over 90% of cases for which $\Delta\tilde{\delta}_{HN}/\Delta T > -4.6$ ppb/K the amide in question participates in an intra-molecular hydrogen bond (Cierpicki & Otlewski, 2001; Cierpicki et al., 2002). Conversely, for the majority of amide protons for which $\Delta\tilde{\delta}_{HN}/\Delta T < -4.6$ ppb/K the amide proton was found to be hydrogen bonded to solvent water. Although the temperature coefficient is a qualitative parameter at best, it nevertheless provides some insight into structure (hydrogen bond) formation even at an early stage in data analysis when only ^{15}N and $^{1}H^{N}$ chemical shifts are generally available. Herein we describe a straightforward approach for obtaining accurate $\Delta\tilde{\delta}_{HN}/\Delta T$ values of excited protein states using relaxation dispersion NMR spectroscopy and provide an illustrative example involving a protein folding system in which a folded (ground) state exchanges with an unfolded, invisible (excited) conformer.

The Pfl6 Cro I58D protein studied here (Pfl6 I58D) is a member of the Cro family of bacteriophage transcription factors with a mixed α+β fold similar to the λ Cro repressor, with the mutation at position 58 ensuring a monomer structure in solution (LeFevre & Cordes, 2003; Roessler et al., 2008). Initial ^{15}N and $^{1}H^{N}$ CPMG relaxation dispersion experiments established that the protein exchanges between two states, $G \underset{k_{EG}}{\overset{k_{GE}}{\rightleftarrows}} E$, with values for $k_{ex} = k_{EG}+k_{GE}$ and the fractional population of the excited state, p_E, of (875±9)s^{-1} and (6.3±0.1)%, respectively (2.5°C). Interestingly, as the temperature is increased p_E decreases - to (3.9±0.1)% at 15°C. The chemical shifts of the excited state that are extracted from fits of the dispersion experiment compare vary favorably with shifts obtained from small ‘random coil peptides’ published by Wishart and coworkers (Wishart et al., 1995), indicating that this state is unfolded, Figure 1. The exchange reaction studied is thus the result of the cold denaturation of Pfl6 I58D with ‘G’ the native conformation of the protein and ‘E’ an ensemble of disordered states that interconvert very rapidly on the NMR chemical shift time-scale. In order to assess whether the cold denatured state of Pfl6 I58D is completely ‘unfolded-like’, similar to small, unstructured peptides in solution or whether there are ‘pockets’ of structure one could, of course, measure additional dispersion profiles using $^{13}C^{\alpha}$ or $^{1}H^{\alpha}$ probes. Alternatively, we are interested here in evaluating whether insight can be obtained from $\Delta\tilde{\delta}_{HN}/\Delta T$ values that are available from the temperature dependent relaxation dispersion series that has already been performed.

Figure 2 shows $^{1}H^{N}$ dispersion profiles recorded at a static magnetic field of 18.8 T, $R_{2,eff}(\upsilon_{CPMG})$, as a function of temperature for Asn 26 and Leu 44 of Pfl6 I58D. Profiles for all of the residues obtained at 11.7 and 18.8 T were fitted together, with separate k_{ex} and p_E for each temperature and with the difference in chemical shifts between an amide proton in states E and G, $\Delta\tilde{\omega}_{HN}=\tilde{\delta}^{E}_{HN} - \tilde{\delta}^{G}_{HN}$, assumed to vary linearly with temperature. The fits so obtained for Asn 26 and Leu 44 are shown with solid lines in the Figure. Only $|\Delta\omega_{HN}|$ values are obtained from analysis of relaxation dispersion data and the sign of the shift difference is critical for calculation of excited state temperature coefficients (see below). Signs of $\Delta\tilde{\omega}_{HN}$ were obtained from (i) a comparison of the amide proton resonance positions measured from (ground state) peaks in ^{15}N-$^{1}H^{N}$ HSQC spectra recorded at 11.7 and 18.8 T (Bouvignies et

al., 2010) and (ii) from a comparison of $^{1}H^{N}$-^{15}N zero- and double quantum CPMG relaxation dispersion profiles in cases where the signs of ^{15}N $\Delta\omega$ values were available (Orekhov et al., 2004).

Figure 3 shows the temperature dependence of the ground state amide chemical shifts for Asn 26 and Leu 44 of Pfl6 I58D (green circles) obtained from ^{15}N-$^{1}H^{N}$ correlation spectra (insets). For a system that is not in exchange the temperature coefficient (of the ground state amide proton) would be calculated simply as the slope of the shift *vs.* temperature profile. However, exchange leads to a small perturbation in the position of the peaks derived from exchanging spins, $\tilde{\delta}_{ex}$. In the case where $p_G \gg p_E$ it has been shown that $\tilde{\delta}_{ex}=k_{GE}\,\xi_{HN}/\left((1+\rho)^2+\xi_{HN}^2\right)$, where $\xi_{HN}=\Delta\tilde{\omega}_{HN}/k_{EG}$, $\rho=\Delta R_2/k_{EG}$ and $\Delta R_2=R_2^E-R_2^G$ is the difference in intrinsic transverse relaxation rates between nuclei in the two states (Anet & Basus, 1978; Skrynnikov et al., 2002). Values of k_{EG} vary from approximately 900 s^{-1} (2.5°C) to 3000 s^{-1} (15°C), with $R_2^G<50$ s^{-1} (11.7 T) so that ρ can be neglected with little error. Using the relation for $\tilde{\delta}_{ex}$ along with the exchange parameters that are isolated from fits of dispersion profiles (Figure 2) the position of the ground state in the absence of exchange ($\tilde{\delta}_{HN}^{G}$) is calculated from the measured chemical shift ($\tilde{\delta}_{HN,measured}^{G}$) as $\tilde{\delta}_{HN}^{G}=\tilde{\delta}_{HN,measured}^{G}-\tilde{\delta}_{ex}$. These values are plotted in Figure 3 (blue circles) along with the best-fit lines from which the amide temperature coefficients in the ground state are calculated, $\Delta\tilde{\delta}_{HN}^{G}/\Delta T$. Once values for $\tilde{\delta}_{HN}^{G}$ are obtained the corresponding chemical shift values in the excited state, ($\tilde{\delta}_{HN}^{E}$) are calculated from the relation $\tilde{\delta}_{HN}^{E}=\tilde{\delta}_{HN}^{G}+\Delta\tilde{\omega}_{HN}$ and the excited state temperature coefficients subsequently generated from the slope of $\tilde{\delta}_{HN}^{E}$ *vs.* temperature, Figure 4.

Figure 5 plots both the ground, folded (blue) and the excited, unfolded (red) state temperature coefficients calculated as described above. It is noteworthy that the dispersion methodology allows for measurements to be made on both states under identical conditions so that $\Delta\tilde{\delta}_{HN}/\Delta T$ values can be properly compared. Not surprisingly a range of values are noted for the ground state with the majority of $\Delta\tilde{\delta}_{HN}^{G}/\Delta T$ values greater than −4.6 ppb/K, the cutoff for intra-molecular hydrogen bonding (Cierpicki & Otlewski, 2001; Cierpicki et al., 2002). For residues at the C-terminus, extending from Arg 56 and beyond the temperature coefficients are centered about −7.5 ppb/K, much lower than for the remaining protein. This provides strong evidence that these residues are unstructured, as expected on the basis of ^{15}N and $^{1}H^{N}$ chemical shifts. An NMR study of a closely related protein, an A33W/F58D/Y26Q triple mutant of the λ Cro repressor that is monomeric in solution, shows that the C-terminal 10 residues of the native state are highly disordered, with no medium or long range NOEs observed in this region and with a very high level of backbone dynamics based on ^{15}N relaxation studies (Newlove et al., 2006). In contrast to residues in the structured region of the folded state (residues 3–55), for which the majority of $\Delta\tilde{\delta}_{HN}/\Delta T$ values exceed −4.6 ppb/K, temperature coefficients for the excited state are in general smaller, consistent with the absence of intra-molecular hydrogen bonding. This is in keeping with expectations for a denatured ensemble of conformers. Interestingly, however there are a (small) number of residues with larger temperature coefficients, including Gln 27, Ser 28 and Asp 47, Gly 48 and Arg 49 that potentially indicate some level of structure even in the denatured state. These ‘anomalous’ $\Delta\tilde{\delta}_{HN}/\Delta T$ values are not artifactual; high quality fits of the dispersion profiles and the resultant $\tilde{\delta}_{HN}^{G}$ *vs.* temperature curves are obtained in these cases (Figure S1 of Supporting Information). Notably, however, the ^{15}N and $^{1}H^{N}$ excited state chemical shifts for these residues fall within the range expected for denatured proteins, suggesting that, at

least in some cases, temperature coefficients may provide a more sensitive measure of residual structure. In native Pfl6, residues 27 and 28 form the center of a motif (residues 24–29; VNQSAI) that caps helix 3 at its N-terminus. The sequence and structure of this region partially resemble a classic helix capping box (Seale et al., 1994), a stable initiation motif that could retain partial order even in the absence of the global tertiary fold. Similarly, residues 47–49 form a β-turn in the folded state (Newlove et al., 2006). It may well be that such a turn is present – at least partially – in the unfolded state as well, serving to initiate the native state β-sheet that is formed in this region. A more quantitative description must await further relaxation dispersion studies focusing on measurement of $^{1}H^{\alpha}$ as well as ^{13}C backbone and $^{13}C^{\beta}$ chemical shifts that are powerful indicators of secondary structure (Wishart & Case, 2001).

Further insight into the nature of the cold denatured ensemble of Pfl6 I58D can be obtained by comparing the distribution of amide proton temperature coefficients for this state with those obtained for both the folded conformer (minus the C-terminus) and the C-terminal region of the folded state that is highly disordered. Figure 6 plots the three distributions that have been normalized so that each has the same area. Notably $\Delta\tilde{\delta}_{HN}/\Delta T$ values for the cold denatured state are centered between those of the well-folded native state conformer and a random coil peptide corresponding to the C-terminal end of the folded protein. The wide distribution of temperature coefficients for the excited state suggests that the structure of the cold denatured state does not resemble a highly dynamic, random coil chain. Studies by Merutka *et al.* (1995) using a disordered small linear peptide model system –GGXGG– showed that values of $\Delta\tilde{\delta}_{HN}/\Delta T$ ranged from −6.4 (Asp) to −9.3 ppb/K (Tyr) with a median(±std) of −7.65±0.68 ppb/K over all 20 amino acids, X. The cold denatured state of Pfl6 I58D shows a much larger range than for these peptides (Figure 6, red curve). This is consistent with other studies of unfolded protein states, such as the unfolded ensemble of the N-terminal SH3 domain from the protein drk (Crowhurst & Forman-Kay, 2003; Zhang & Forman-Kay, 1997), which clearly shows regions with structure, including non-native interactions, despite the fact that the protein is ‘unfolded’.

In summary, we have presented a CPMG relaxation dispersion approach for measuring amide proton temperature coefficients of excited protein states. Because both ground and excited conformers are present in solution it becomes possible to measure temperature coefficients of both states under identical conditions so that they can be readily compared. The methodology has been applied to the study of the cold denatured ensemble of Pfl6 I58D where $\Delta\tilde{\delta}_{HN}/\Delta T$ values are for the most part significantly more negative than for the folded conformer and less than −4.6ppb/K, consistent with the absence of intra-molecular hydrogen bonds, on average. Nevertheless, temperature coefficients are not uniform and span a significant range (5.6±2.1 ppb/K), suggesting that the unfolded state does not approximate a random coil chain, but like other unfolded proteins, samples a distribution of transiently formed conformers. The methodology presented adds to a growing set of experiments for characterizing small transient populations of proteins under ‘native-like’ conditions that can provide a detailed atomic resolution description of conformations that are recalcitrant to study using other techniques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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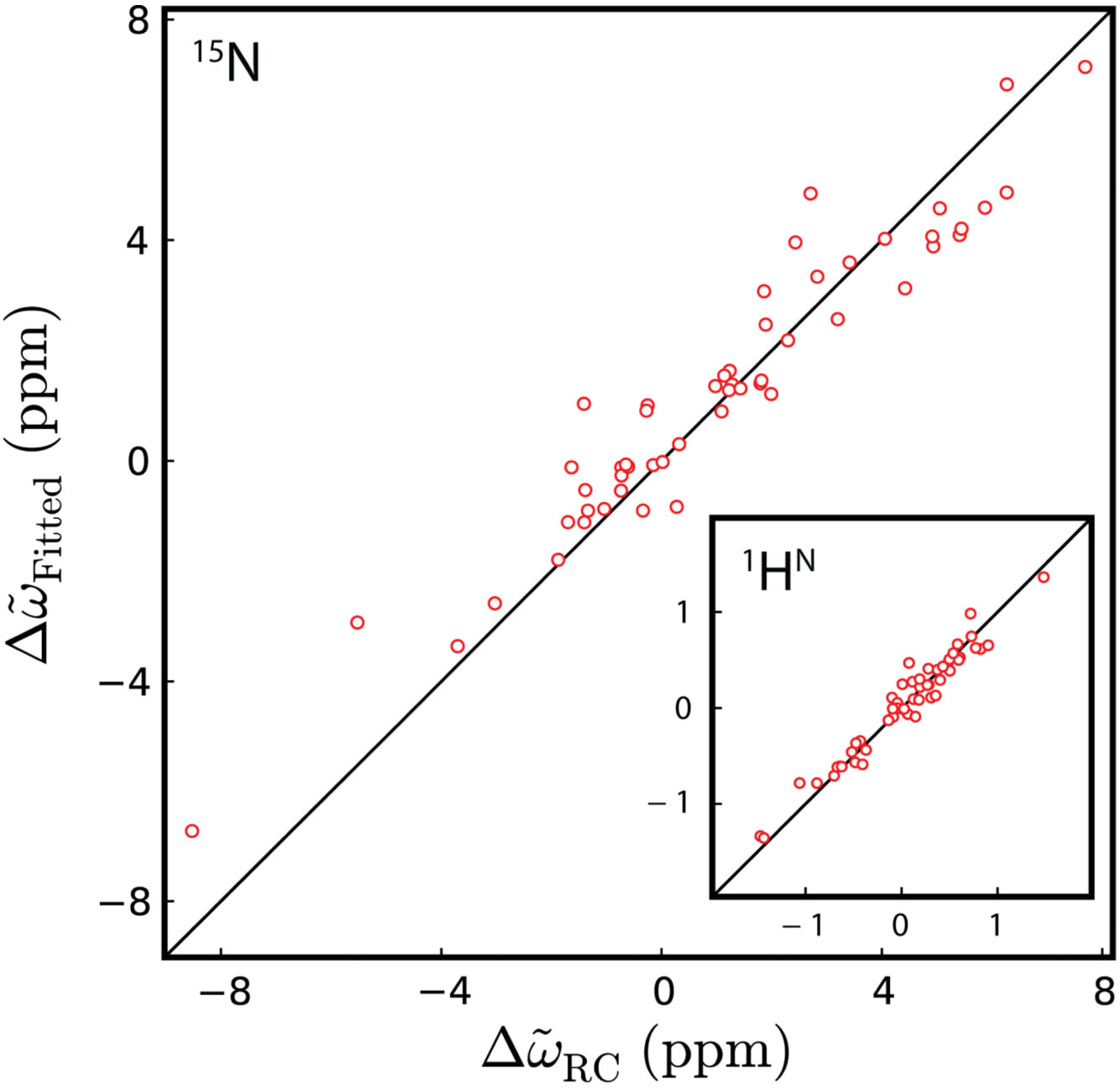

Figure 1.

Correlation plot of ^{15}N (^{1}H^{N}, inset) chemical shift differences between ground and excited states of Pfl6 I58D ($\Delta\tilde{\omega}_{Fitted}$) measured by relaxation dispersion experiments (5°C) and predicted shift differences assuming that the excited state is unfolded ($\Delta\tilde{\omega}_{RC}$) calculated as described by Wishart and coworkers (Wishart et al., 1995). The RMSDs of the ^{15}N and ^{1}H^{N} correlations are 0.95 and 0.13 ppm, respectively.

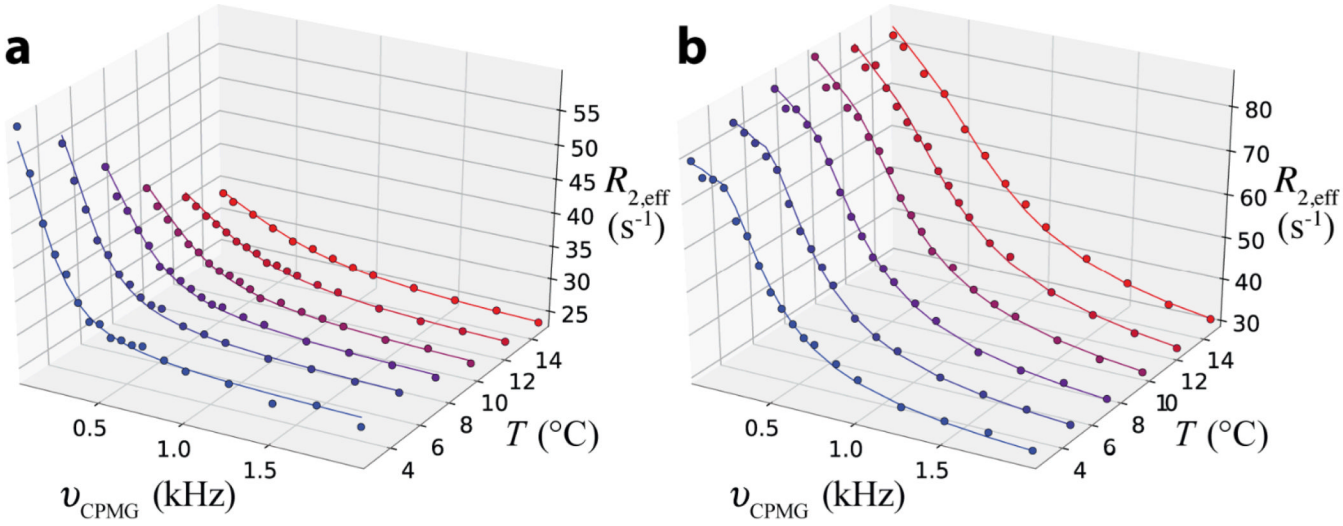

Figure 2.

Experimental $^{1}H^{N}$ dispersion profiles (circles) for Asn 26 (a) and Leu 44 (b) of Pfl6 I58D measured at temperatures (T) ranging from 2.5°C (blue) to 15.0°C (red), along with the best fit dispersion profiles (solid line). Dispersion profiles were fit to models assuming (i) $\Delta R_2 = 0$ or (ii) $\Delta R_2 = -\frac{R_2^G}{2} (i.e., R_2^E = \frac{R_2^G}{2})$. The slope of the linear correlation of the resulting temperature coefficients of the excited state obtained using both approaches is 0.98 with a pair-wise rmsd of 0.2 ppb/K, indicating that for this application the results are not sensitive to the values of ΔR_2.

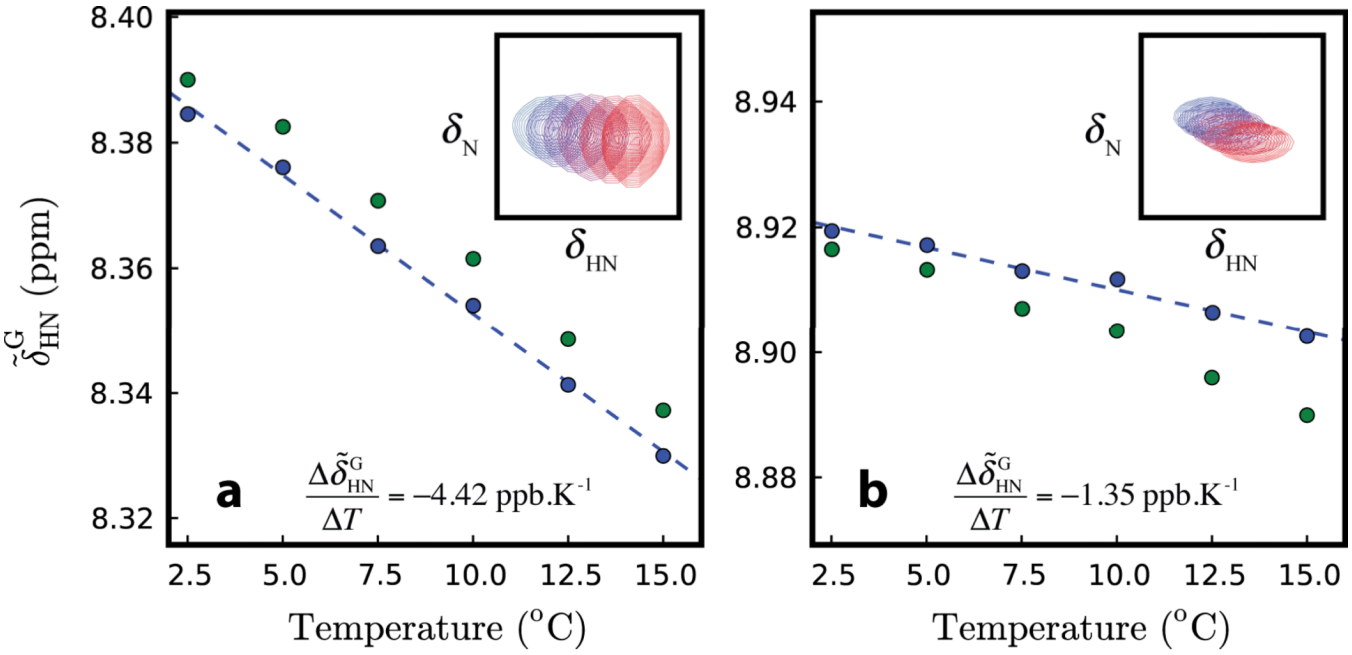

Figure 3.

Ground state $^1H^N$ chemical shifts ($\tilde{\delta}^{G}_{HN}$) *vs.* temperature for Asn 26 (a) and Leu 44 (b) of Pfl6 I58D. Shifts measured directly from spectra are plotted in green; they are then corrected for exchange that 'moves' the ground and excited state correlations towards each other and subsequently replotted in blue (see text). The dashed lines are the best linear fits to the 'corrected' peak positions from which the ground state $^1H^N$ temperature coefficients are extracted. Insets show the corresponding $^1H^N$-^{15}N correlation peaks from spectra recorded at temperatures ranging from 2.5°C (blue) to 15.0°C (red).

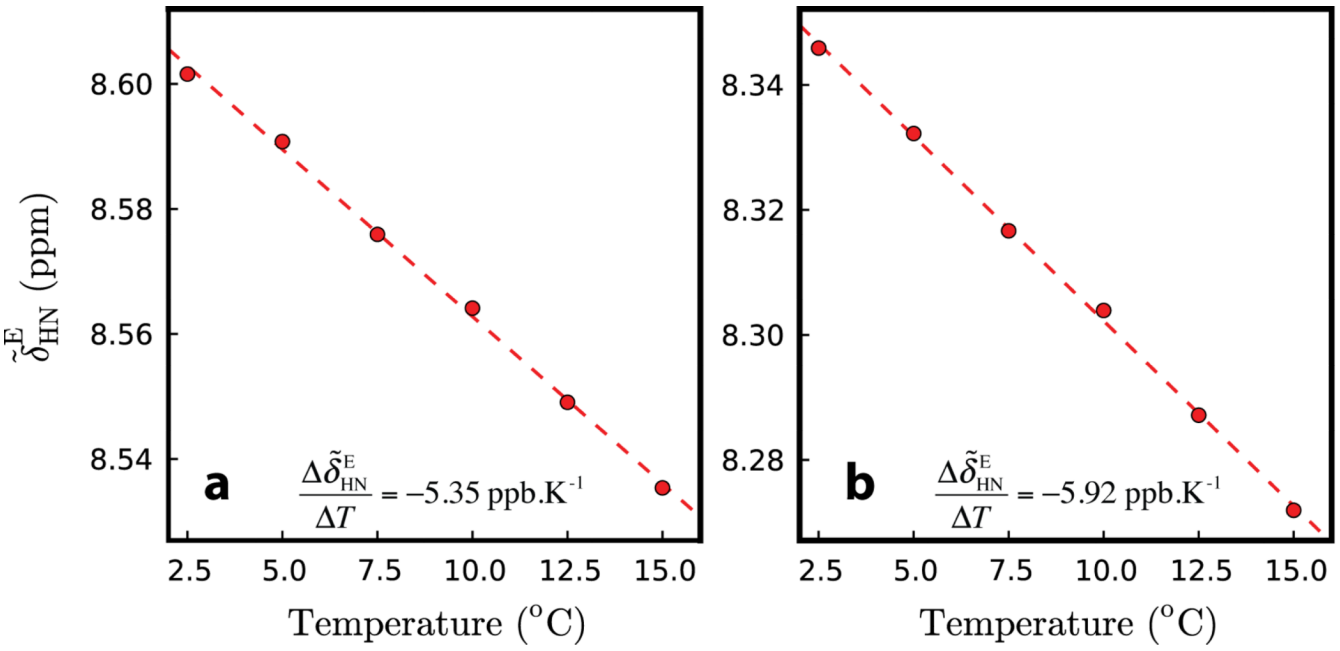

Figure 4.

Excited state $^{1}H^{N}$ chemical shifts ($\tilde{\delta}^{E}_{HN}$) *vs.* temperature for Asn 26 (a) and Leu 44 (b) of Pfl6 I58D. Note that Asn 26 and Leu 44 have different temperature coefficients in the ground state yet similar values in the excited state.

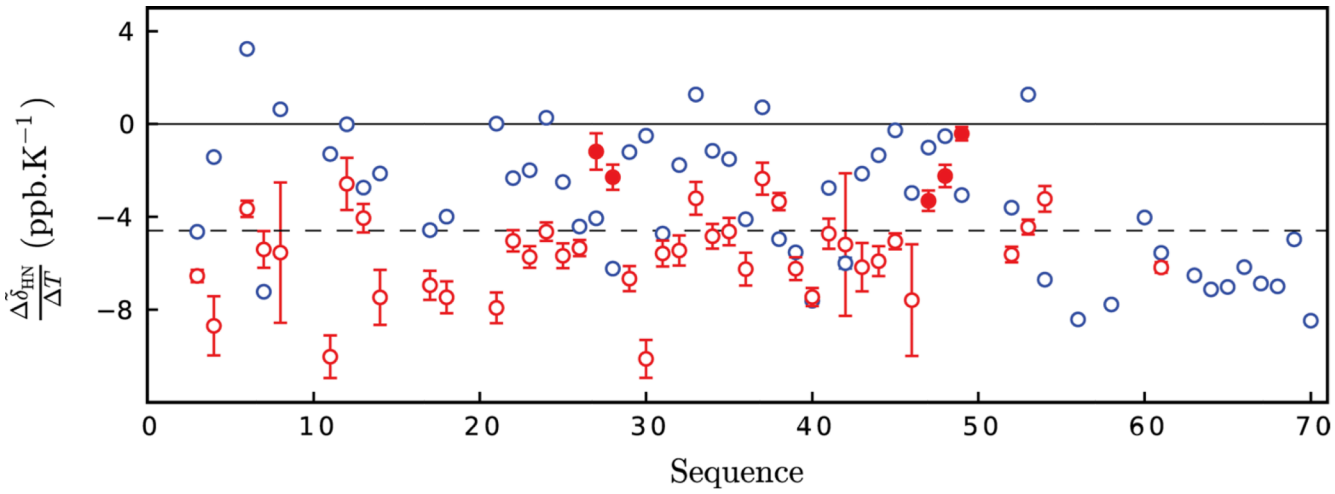

Figure 5.

Ground (blue) and excited (red) state temperature coefficients $\Delta\tilde{\delta}_{HN}/\Delta T$ for Pfl6 I58D as a function of primary sequence. The dashed line is plotted at −4.6 ppb/K, the cut-off used to establish whether amide protons participate in intra-molecular hydrogen-bonding (Cierpicki & Otlewski, 2001; Cierpicki et al., 2002). Excited state temperature coefficients for residues 27,28 and 47–49 highlighted in the text are indicated by filled circles. Temperature coefficients are reported only in cases where signs of $\Delta\tilde{\omega}$ values could be determined.

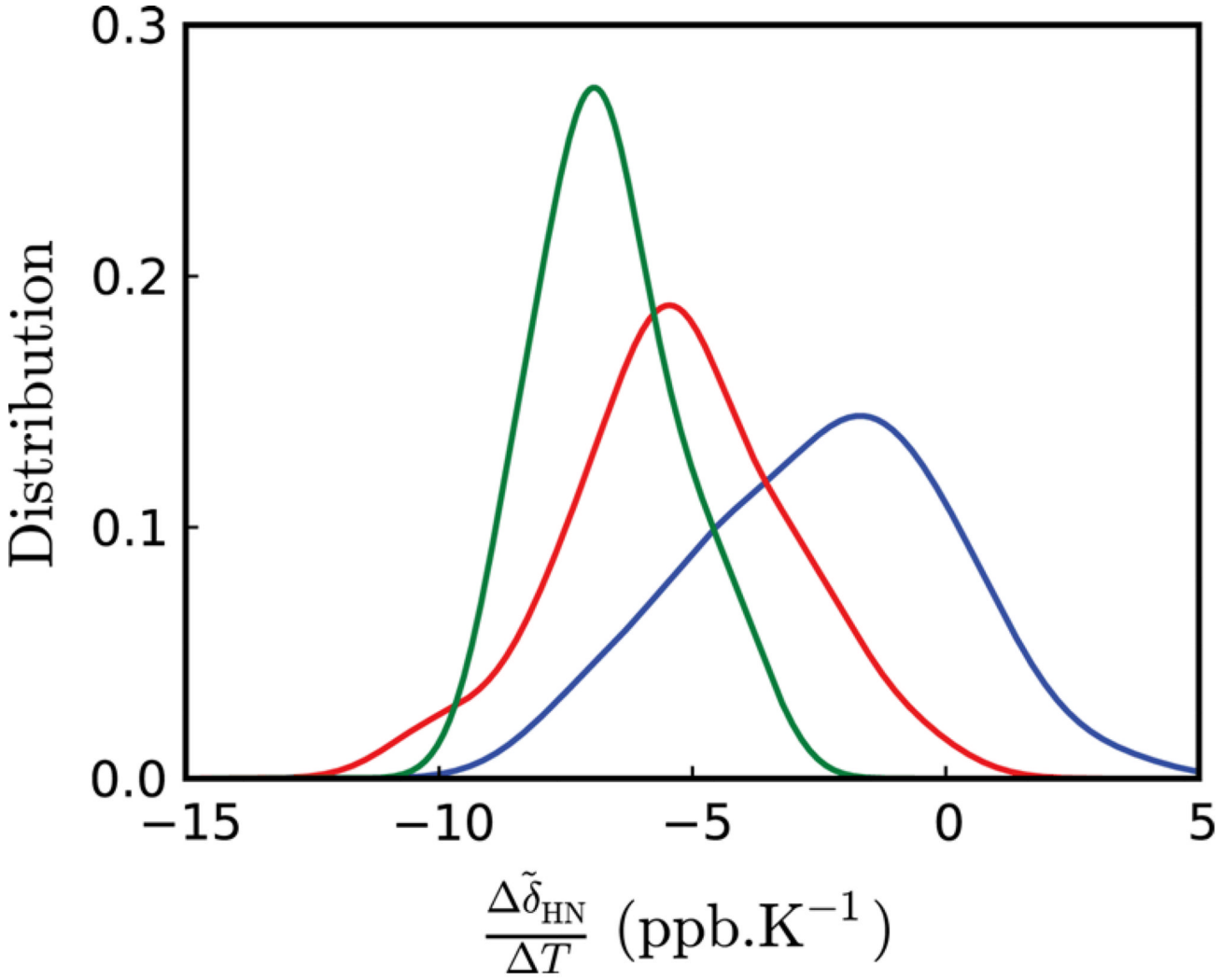

Figure 6.
Normalized distributions of $^{1}H^{N}$ temperature coefficient values for the ground (blue, minus the C-terminus) and excited (red) states of Pfl6 I58D; the disordered C-terminal region of the ground state is shown in green. The distributions were obtained using a Gaussian kernel density estimation (Scott, 1992).