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β-Sheet ^{13}C Structuring Shifts Appear Only at the H-Bonded Sites of Hairpins

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Supporting Information

ABSTRACT: The ^{13}C chemical shifts measured for designed β-hairpins indicate that the structuring shifts (upfield for Cα and C′, downfield for Cβ) previously reported as diagnostic for β-structuring in proteins appear only at the H-bonded strand residues. The resulting periodicity of structuring shift magnitudes is not, however, a consequence of H-bonding status; rather, it reflects a previously unrecognized alternation in the backbone torsion angles of β-strands. This feature of hairpins is also likely to be present in proteins. The study provides reference values for the expectation shifts for ^{13}C sites in β-structures that should prove useful in the characterization of the folding equilibria of β-sheet models.

Over the past 15 years, β-hairpins have been developed as models of β-sheet structures in proteins.[1−5] Until very recently,[6−9] the fold stabilities of designed hairpins were marginal: the fold populations in water at ambient temperature rarely exceeded 80%. With hairpin dynamics in the 1−50 μs range,[10,11] which leads to population-weighted chemical shifts, chemical shift deviations (CSDs) from random coil values have emerged as the primary tool for determining hairpin fold populations. Since 1999, we have advocated the use of the larger CSDs (ca. 1 ppm) of the cross-strand directed Hα sites in non-H-bonded strand sites and those of the H_N's of the H-bonded sites for estimating hairpin fold populations.[12] Detailed studies of hairpins and correlations with shifts in the edge strands of protein β-sheets have established that the CSDs of backbone NHs and Hα's do, indeed, display alternating magnitudes along β-strands.[13,14] Although the ^{13}C CSDs for residues in protein β-sheets are even larger, there do not appear to have been any reports suggesting a similar differentiation of ^{13}C CSDs in β-structures. The present study of hairpins was undertaken to look for differences in structuring shifts for H-bonded (HB) versus non-H-bonded (NHB) sites in hairpins.

The directions of the structuring shifts associated with β-structuring, upfield for ^{13}C═O and ^{13}Cα versus downfield for ^{13}Cβ, have been known at least since 1991,[15,16] and these have been widely used to assign protein secondary structure. Computational studies suggest that these reflect predominantly the distinct ϕ/ψ torsion angles in helical versus β-structures.[17,18] In 2008, Vila and Scheraga[19] classified nuclei in proteins in order of the usefulness of their CSDs in elucidating secondary structure; for β-structure definition, the following sequence was given: ^{1}Hα > ^{13}Cβ > $^{1}H_N \approx {}^{13}$Cα ≈ ^{13}C═O ≈ ^{15}N. Given the larger structuring shifts associated with ^{13}C nuclei, and the general expectation that ring current shifts should be less significant for ^{13}C CSDs,[20,21] this statistical observation is somewhat surprising and was another impetus for the studies reported herein. The present study indicates that the diagnostic ^{13}C═O, ^{13}Cα, and ^{13}Cβ CSDs for β-structuring are associated, almost exclusively, with the HB residues and, in the case of ^{13}C═O shifts, run counter the prior expectation based on both experimental studies of H-bonding effects[22] and density functional theory (DFT) calculations[18] at the B3LYP/6−31G** basis set level for β-models.

The dominating downfield shifts of ^{13}Cα, and upfield shifts of ^{13}Cβ, at HB sites of designed hairpins are illustrated in Figure 1. We selected the well-studied MrH hairpin[3,12] series (KKLTV-IXGK-KITVSA analogs, for which proton CSD methods for assessing the extent of folding have been validated[14,23,24]) to illustrate the trend in ^{13}C CSDs along the strands. The X = Asn to

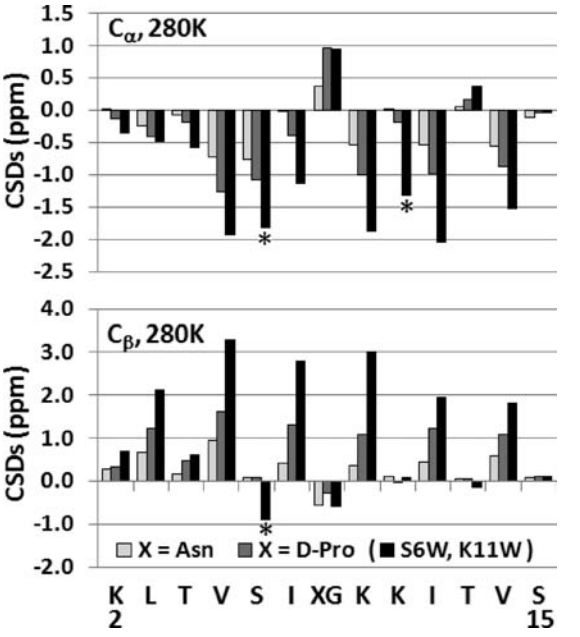

Figure 1. ^{13}C CSD values along the sequence of a series of hairpin analogs demonstrating a fold population associated increase in magnitude for the HB sites (L3, V5, S7, K10, I12, and V14). The asterisks indicate CSDs that, in part, reflect ring current effects associated with the added Trp residues: upfield for the Cα within a Trp residue in a β-configuration, and (lower panel) upfield at Cβ of the edge Trp in an edge-to-face cross-strand indole/indole interaction geometry.[24]

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D-Pro mutation increases hairpin fold stability, and this is reflected by comparable magnitude increases in the CSDs for both the Cα and Cβ CSDs at the HB sites. An (S6W,K11W)-double-mutation results in additional fold stabilization and further increases in the CSD magnitudes (Figure 1).

A recently reported series of hairpins, stabilized by a capping interaction at the hairpin termini,[9] provides a confirmation of the HB site specificity of β-structuring shifts: Ac-WITVT-IHGK-KIRVWTG-NH_2 displays Cα CSDs of -1.5 ± 0.6 and Cβ CSDs of $+2.8 \pm 0.5$ ppm at the HB sites (underlined) with the adjacent NHB site Cα/Cβ CSDs not significantly different from zero (-0.2 ± 0.5 ppm). Further details and examples appear in the Supporting Information. In follow up studies of additional hairpins, these trends continue to be observed; for example, in the current set of Cβ CSDs, corrected to 100% folding as needed (162 Cβ sites, nearly equally distributed between HB and NHB) the mean and standard errors are $+2.64 \pm 0.93$ (HB sites) versus $+0.42 \pm 0.65$ (NHB sites). As a result, we recommend using Cβ CSDs of HB sites as an alternative measure of hairpin formation. In this regard, we note that Santiveri et al.[21,25] have provided guidelines for using $^{13}C\beta$ and $^{13}C\alpha$ CSDs for hairpin fold stability estimation (based on $+1.95$ and -1.55 ppm shifts, respectively, for 100% folding *with averaging over all non-terminal strand sites*).

Backbone carbonyl shifts are more difficult to access, but the isotopic substitutions required[26] for such studies appeared to be worth it to extend our study to the ^{13}C site that might be expected to display the largest H-bonding effect.[27] Our initial expectation was that a distinct HB versus NHB shift difference would be found, with the cross-strand H-bonding interaction producing a deshielding effect which might nullify the $^{13}C{=}O$ shielding associated with the ϕ/ψ torsion angles in β-strands. Thus we expected the NHB positions to display the larger upfield shifts. A series of MrH analogs with two Val $^{13}C{=}O$ labels incorporated into each hairpin, one on each β-strand, selectively at cross-strand HB and NHB positions, were prepared (the complete structures and chemical shifts as well as the control peptides for calculating CSD values appear in the Supporting Information). In addition, $^{13}C'$-Val and -Ala were incorporated in β-capped hairpins at HB and NHB sites (see Supporting Information). The HB sites were further upfield and also the only ones displaying a CSD magnitude melt as the fold population decreases on warming. The mean and standard error for $^{13}C'$ at NHB sites in well-folded hairpins were 0.09 ± 0.34, not significantly different than zero.

The hairpin fold populations of MrH peptides can be altered by both mutations (insertion of alanine in a strand position or altering the turn locus) and fluoroalcohol co-solvent addition. With the additional observations made available from these mutational and media studies as well as at higher temperatures, $^{13}C'$ CSD data spanning the full range of hairpin fold populations were available for species with a pair of $^{13}C'$-Val units at HB sites. The correlation between hairpin fold population (χ_F, derived from Hα CSDs as previously described[14,24]) and the average $^{13}C'$ CSD appears in Figure 2.

The correlation between $^{13}C'$ CSDs for HB sites and the extent of folding is linear with no systematic deviations for possible solvent or temperature (280−340 K) effects. There do appear to be small, but significant, differences in the 100%-folded CSD value depending on the specific placement of the $^{13}C'$-Val probes in the hairpin (see Supporting Information). It is, however, apparent that any of the HB sites examined can serve as a dependable probe of the extent of hairpin formation. There

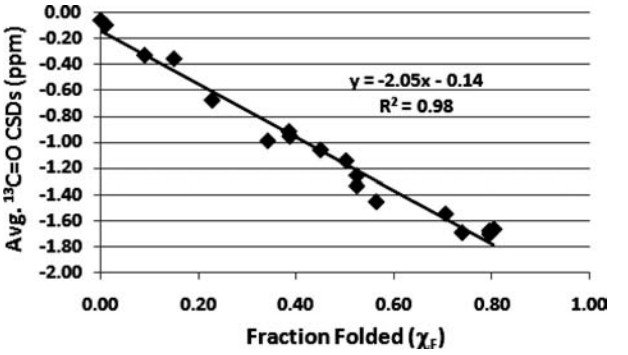

Figure 2. $^{13}C{=}O$ CSDs display a linear correlation with the extent of hairpin folding. The complete set of CSDs and fold estimates at 280 K are tabulated in the Supporting Information.

was no correlation between the extent of folding and the magnitude of the smaller CSDs observed for the NHB $^{13}C{=}O$ sites.

The chemical shift data presented indicate that the C′, Cα, and Cβ chemical shift diagnostics previously associated with being in β-structured regions of proteins appear exclusively at the HB sites of hairpins. These site specific resonances can be added to the cross-strand directed Hα and HN sites as probes of folding and melting in β-sheet models, a field where downhill folding scenarios are under consideration as alternatives to two-state folding.[28] We have adopted CSD values of $+3$, -2.6, and -2.2 for Cβ, C′, and Cα, respectively, as default 100%-folded standards in our continuing effort to assess the extent of folding of β-sheet models. These values apply only for the HB strand sites. While the smaller structuring shifts at Cα and Cβ of NHB sites also melt out on thermal unfolding, they do not display diagnositc values; a more detailed analysis indicates that Coulombic and ring current effects are often the largest contributors to these CSDs. The greater effect of electrostatics on ^{13}C shifts has been noted previously.[22]

In the designed hairpins of the present study, the alternating magnitude of ^{13}C CSDs along β-strands is most distinct for $^{13}C\beta$ and somewhat less dramatic for $^{13}C\alpha$. This observation appears to be consistent with Scheraga's finding[19] that, of the ^{13}C shifts, Cβ provides the best definition of β-structuring. Preliminary surveys of protein ^{13}C shifts (work in progress) suggest that the shift trends observed for hairpins also apply to antiparallel edge strands of protein β-sheets.

The prior literature does not provide a rationale for the sign of the shielding differences which we observe along a β-strand associated with another antiparallel β-strand. Hydrogen bonding has been viewed[27] as a major contributor to the $^{13}C'$ chemical shift differentiation associated with secondary structure motifs. In the case of carbonyl carbons there is a large body of experimental data indicating that H-bonding produces downfield shifts, rather than the upfield increment we observed for the H-bonded sites in hairpins: in fact, downfield shifts associated with short H-bonds as large as $+10$ ppm have been observed.[22,29] An early rationale of the upfield location of β-sheet C′ (versus helical C′ sites) took this effect of H-bonding into account; De Dios and Oldfield suggested[27] that the relatively upfield location of β-sheet C′ does not reflect "direct ϕ,ψ effects, but rather are dominated by strong peptide carbonyl-HN H-bonding in the α-helices". With regard to the downfield C′ shifts in helices, experimental data[30] indicate that at least half of the 3.5 ppm downfield shift for C′ sites at the central residues of peptide helices is associated with the H-bonded state of these sites. DFT calculations[18] of shifts for

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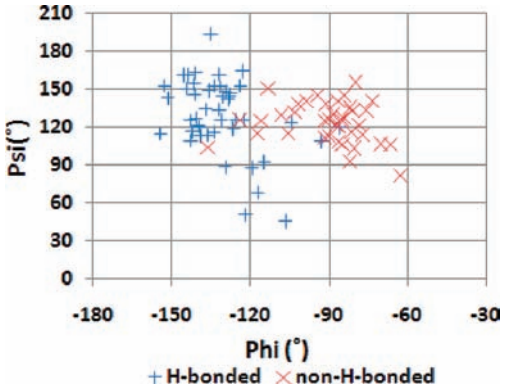

Figure 3. A Ramachandran plot for the strand residues of 11 hairpins demonstrates a differentiation in ϕ/ψ values: with the H-bonded sites close to the center of the β-structure basin and the non-HB sites in the polyproline II basin.[34]

helical models also support this conclusion: for an $(Ala)_9$ model restrained to helical ϕ/ψ values, the central site was 2.2 ppm downfield of the non-H-bonded sites closer to the termini. When the same shift calculations were applied to monomeric and H-bond-associated dimeric and trimeric models of a $(Gly)_7$ β-strand, a 0.9 ppm shift difference results for HB versus NHB C′ sites, with the HB sites downfield. The calculations also predict a 0.8 ppm H-bond-associated shift for Cα sites. Both of these calculated H-bonding effects are in the opposite direction of the shifts we observe in β-hairpin peptides. The greater upfield shift of the H-bonded $^{13}C{=}O$ sites in β-hairpin strands reported herein can not be rationalized based on any of the reported studies.

In the case of Cα and Cβ structuring shifts, essentially all theoretical studies imply that the ϕ/ψ values of the residue are the primary determinants of shift deviations from random coil norms. Recent calculations[31] suggest that β-hairpin ^{13}C structuring shifts may also reflect χ^1 differences. The compilation of amino acid pairing preferences of Hutchinson and co-workers (http://www.rubic.rdg.ac.uk/betapairprefsparallel/) provides access to χ^1 values at NHB versus HB sites in antiparallel strands of proteins; while there are some χ^1 preferences for certain cross-strand amino acid pairings, there are no differences in the overall χ^1 preferences (g+ > g− > t) for HB versus NHB sites. To our knowledge, there have been no reports noting ϕ/ψ differences for NH and NHB sites in antiparallel protein β-sheets. As a result, we turned to high resolution NMR structures of β-hairpins to look for possible correlations: we found 11 structures, 4 from our prior studies[9,24,32] and 7 from the literature.[33] The g+ conformation is also preferred at both the NHB (61%) and HB (68%) sites of these hairpins. The relative amounts of the minor t and g− χ^1 conformations are different, but this is judged unlikely to be the source of the ^{13}C shift trends observed. A rationale was, however, immediately apparent from the Ramachandran plot for the strand residues of these β-hairpin structures (Figure 3).

Based on the ^{13}C shift dependence of A2 and A3 (in GAAAG) on the ϕ/ψ values of A3 presented by Case,[18] both the downfield shift of Cβ and the upfield shift of Cα are maximal at $\phi \approx -130°$, with a very rapid decrease in the CSD magnitude predicted as ϕ moves toward −80°. The calculated shift changes are in near-perfect agreement with the CSD magnitude changes we observe for HB versus NHB sites in hairpins. In the case of $^{13}C{=}O$, the effect observed can also be rationalized as the result of the ϕ/ψ variation seen in Figure 3. The $^{13}C'$ shielding increases associated with the ϕ/ψ changes at the NHB sites, combined with a ≥1 ppm deshielding at HB sites due to the less negative ϕ value at the following NHB site, account for the CSD difference observed. We conclude that ϕ/ψ torsion angles are indeed, the primary basis of variations in the structuring shifts of all three ^{13}C sites examined.

With the present findings taken into account, ^{13}C CSDs should prove to be excellent probes of hairpin fold stability and unfolding pathways[28] and can provide details of β-sheet geometry changes in proteins. We expect that ^{13}C CSDs will provide more dependable measures of structure than ^{1}H CSDs. In the case of hairpin folding studies, we suggest that only the ^{13}C structuring shifts of the HB sites should be used for fold assessment.

■ ASSOCIATED CONTENT

Supporting Information. Sample preparation, data collection and CSD calculation methods are detailed, peptide sequences and the observed $^{13}C'$ CSDs are tabulated, and a figure showing ^{13}C shifts in β-capped hairpins. This material is available free of charge via the Internet at http://pubs.acs.org.

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