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Leucine Side-Chain Conformation and Dynamics in Proteins from ^{13}C NMR Chemical Shifts

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The γ-gauche effect in ^{13}C NMR spectroscopy refers to the magnetic nonequivalence of the methyl carbons of the terminal isopropyl groups that are attached to branched alkanes;[1–3] this effect results in magnetic shielding of the carbon nucleus of the substituent in the *gauche* position by about 5 ppm relative to that of the same chemical group in the *trans* position.[4–6] Tonelli and colleagues demonstrated that the ^{13}C chemical shift of a carbon nucleus in a particular polymer stereoisomer is attributable solely to stereosequence-dependent differences in the probability that the given carbon atom is involved in three-bond gauche interactions with other heavy atoms. Through this simple observation they were able to accurately and quantitatively predict the experimental ^{13}C NMR spectra of polypropylene and polypropylene model compounds with different stereoregularities.[4,5,7,8]

We demonstrate here the use of the ^{13}C NMR γ-gauche effect to establish leucine rotamer conformations in proteins, and provide a quantitative measure of their dynamics. This information is of value as a restraint on side-chain conformation in protein structure model building,[9,10] and is highly valuable for the interpretation of side chain methyl dynamics from ^{2}H or ^{13}C nuclear spin relaxation.[11–16] Although we focus below on methyl groups of leucine (Leu) residues, the γ-gauche effect is a general determinant of chemical shifts of amino acid side-chain carbon nuclei, and expected to play an import role in their conformational analysis.[17–19]

Leucine side chains can assume three stable staggered conformations of lowest potential energy as a function of the dihedral angle χ^2, referred to[20] as *gauche*(+) or p, *gauche*(−) or m, and *trans* or t (Figure 1). A carbon, rather than a proton, substituent in the gauche position leads to high internal energy, and results in the prevalence of conformations in which one atom is *trans* to the Cα atom, while the remaining atom is positioned *gauche*. Consequently, the two dominant χ^2 rotamers are t and p. The energetics are mirrored by the χ^2 side-chain distributions found in protein crystal structures.[20,21] In fact, at ambient temperature a small preference (2:1) of t over p is noticed in protein structures, as is observed for branched alkanes.[7] An analysis of the stereospecifically assigned methyl ^{13}C chemical shifts in the BioMagResBank (http://www.bmrb.wisc.edu/) is shown in Figure 2. The NMR data show that the preference of t over p observed in crystalline proteins is perfectly mirrored in solution.

We have previously noted a strong correlation between the $^{3}J_{CC}$ coupling and the methyl carbon chemical shift difference

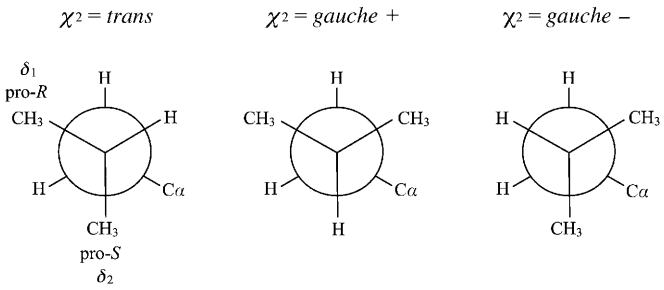

Figure 1. Newman projections of Leu χ^2 side-chain conformations.

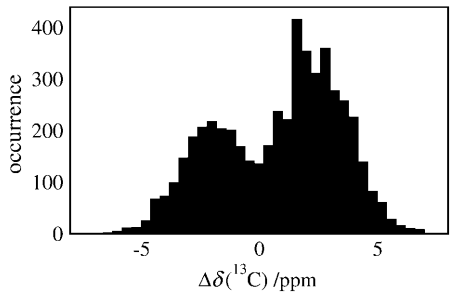

Figure 2. Histogram of Leu chemical shift differences $\Delta\delta(^{13}C) = {}^{13}C(\delta_1) - {}^{13}C(\delta_2)$ for all stereospecifically assigned entries in the BioMagResBank (http://www.bmrb.wisc.edu/). A spike observed in the untrimmed dataset at 0 ppm was eliminated by disallowing those entries for which the two methyl correlations were reported to coincide (those in which $^{1}H(\delta_1) - {}^{1}H(\delta_2) = 0$ ppm).

for leucine residues in two proteins;[22] this suggests that the wide distribution seen in Figure 2 could result from rotameric interconversion about the χ^2 dihedral angle. A recent report by London and co-workers[19] supports this conclusion, and demonstrates that correlations between NMR side chain chemical shifts in solution and single lowest-energy structures modelled from X-ray crystallographic data are compromised by dynamics. Weak correlations are expected whenever several near-energy states about side chain dihedral angles contribute to the experimental NMR observables, but not to model building. The ability to use chemical shifts as restraints on side-chain conformations can therefore only be reliable if knowledge of the extent of dynamic averaging is taken explicitly into account. To this end we have used three-bond carbon–carbon coupling constants, $^{3}J_{CC}$, because chemical shifts and coupling constants are averaged over the same time window, from picoseconds to milliseconds. Leu side-chain conformations and dynamics were derived from $^{3}J_{CC}$ values, by using the long-range "quantitative J" experiment by Bax.[23] That chemical shielding and coupling constants are strongly correlated is validated with data obtained for the 75-residue protein calbindin D_{9k}

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(P43G[24]) with bound calcium: Leucine residues that are in one dominant staggered rotameric state give rise to large (~4 Hz) $^3J_{CC}$ for the methyl group that is in the *trans* position, and small (~0.5 Hz) $^3J_{CC}$ for methyl groups *gauche* relative to Cα. Jumps about χ^2—between p and t conformations—will result in averaging of the measured coupling constants for the two diastereotopic methyl groups to yield $^3J_{CC} \approx 2.2$ Hz. Figure 3

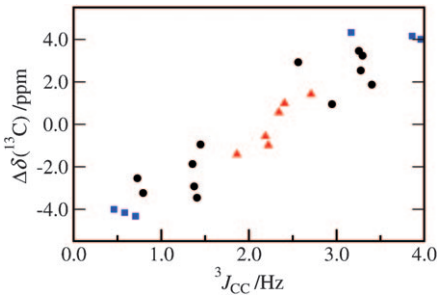

Figure 3. Correlation of the magnitude of the three-bond $^{13}C\delta$-$^{13}C\delta$ scalar coupling with the ^{13}C chemical shifts differences for the two diastereotopic methyl groups in leucine residues of calbindin D_{9k}. As there is only a single chemical shift difference for two methyl groups, but a coupling constant obtained for both, the large coupling is plotted at the ordinate for the absolute difference $|\Delta\delta|$, and the small coupling is plotted at an ordinate $-|\Delta\delta|$.

demonstrates this fact clearly: Leu23, 28 and 31 all occur in a single staggered conformation, and give rise to one large (*trans*) and one small (*gauche*) coupling (blue squares); rotamer interconversions about χ^2 for Leu30, Leu39 and Leu49 lead to averaging of the $^3J_{CC}$ coupling constant and of the methyl carbon chemical shift difference (red triangles). The six remaining leucines (6, 32, 40, 46, 53, 69) demonstrate an intermediate pattern of flexibility (black circles). Linear regression of the data in Figure 3 yields the following relation [Eq. (1)]:

$$\Delta\delta(^{13}C) = -5.5 + 2.5 \times {}^3J_{CC} \qquad (1)$$

with a regression coefficient of 0.95. Evidently ^{13}C chemical shift differences can serve as reporters of the conformation and dynamics of Leu side chain as well as three-bond *J* couplings.

An imminent problem with coupling constants is that an intermediate value can arise from two possible origins: The side chain is statically positioned at a noncanonical angle, or it is undergoing dynamics. To assert the pertaining situation we performed temperature-dependent measurements: If the coupling constant results from transitions between conformations with different free energies, dynamics will generally lead to averaging of the coupling constant with temperature. We noticed this behaviour for calbindin D_{9k}, but found that the changes were small (0.17 ± 0.16 Hz over a 40 °C interval), and at the limit of accurate detection. Since chemical shifts can be measured with far greater precision and in less time than coupling constants in proteins, and we have established that these parameters are correlated, we explored the use of temperature-dependent chemical shift differences as an alternative probe for the conformational dynamics of Leu side chains in calbindin D_{9k}.

The temperature dependence of the ^{13}C chemical shift difference between the diastereotopic methyl groups of leucine residues in calbindin D_{9k} is shown in Figure 4. Most leucines (ex-

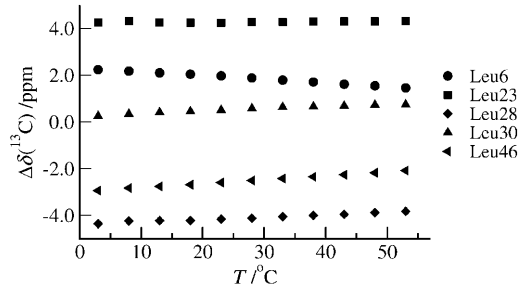

Figure 4. Chemical shift differences $^{13}C\delta_1 - {}^{13}C\delta_2$ for the diastereotopic methyl groups of Leu residues of calbindin D_{9k} as a function of temperature. For clarity only data for a selection of residues is shown.

emplified here by Leu6 and Leu46) show a constant decrease in $|\Delta\delta|$ with increasing temperature; this indicates that the increasing population of higher energy conformations. However, some leucines (Leu23 in Figure 4) exhibit no change with temperature, and this indicates that these side chains are confined to a single rotamer ("staggered") over the entire interval. Leu28 has a single conformation at temperatures below approximately 20 °C, but increasingly populates alternative states at higher temperature. The chemical shift differences for all leucines tend towards 1–1.5 ppm at high temperature; this indicates a persistent preference for t over p conformations, dictated by the internal energy of side-chain conformation. The values for $\Delta\delta(^{13}C)$ in Figure 2 and 4 are consistent with a γ-gauche effect of −5 ppm for methyl groups in Leu. This is in good agreement with results found for isopropyl groups in branched alkanes.[7] For a leucine methyl group that jumps between states that are *trans* and *gauche* relative to Cα, having probabilities P_t and $P_g = 1 - P_t$, respectively, the following relationship holds for the observed carbon chemical shift difference:

$$\Delta\delta(^{13}C) = {}^{13}C(\delta_1) - {}^{13}C(\delta_2) = -5 + 10 \times P_t \qquad (2)$$

In Table 1 the chemical shift and coupling data are compared with methyl axis order parameters derived from deuterium relaxation rates.[25] These data are very similar to those determined for the P43M mutant of the same protein.[26] The presence of averaging about axes between the methyl group and the backbone on the subnanosecond time scale is expected to lead to reduced order parameters, and inconsistency with simple motional models that account for internal correlation functions that decay on the picosecond time scale exclusively.[12] A two-parameter fit with a high methyl axis order parameter was obtained for Leu23 and Leu28 and is indicative of the absence of rotameric transitions. In contrast, data for Leu6, 30, 46 and 69 required a reduced effective rotational correlation time in the fitting. As described for other proteins[12,14,26] this is the hallmark of rotameric transitions taking place on a time scale comparable to molecular reorientation. The pres-

Table 1. Summary of experimental structural and dynamic data for all leucine residues in calbindin D_{9k} at 28 °C.

Leucine residue number	$^3J_{CC}$ (Hz) for $^{13}C\delta_1/^{13}C\delta_2$	$\Delta\delta(^{13}C)=(\delta_1-\delta_2)$ [ppm]	P_t derived from Eq. (2)	χ^2 rotamer in NMR structures 1B1G[24]	χ^2 rotamer in X-ray structure 4ICB[27]	Methyl axis order parameters S^2 for $^{13}C\delta_1/^{13}C\delta_2$	χ^1 averaging?
6	3.4/1.4	1.9	0.69	*trans*	*gauche*(+)	–/0.48	no
23	3.2/0.7	4.3	0.93	*trans*	*trans*	0.72/0.77	no
28	0.6/3.9	−4.2	0.09	*gauche*(+)	*gauche*(+)	0.79/0.82	no
30	2.2/2.3	0.6	0.56	*trans*	*trans/gauche*(+)	0.58/0.61	yes
31	4.0/0.5	3.9	0.89	*trans*	*trans*	–/0.50	no
32	3.3/0.8	3.2	0.82	*trans*	*trans*	–/0.49	no
39	2.4/2.2	1.0	0.60	*trans*	*trans*	0.17/0.18	yes
40	2.6/1.4	2.9	0.79	*trans*	*trans*	–/0.36	no
46	0.7/3.3	−2.5	0.25	*gauche*(+)	*gauche*(+)	0.56/–	no
49	1.9/2.7	−1.4	0.36	*gauche*(+)	*gauche*(+)	0.31/0.22	yes
53	3.3/1.4	3.4	0.84	*trans*	*trans*	–/0.51	no
69	1.4/2.9	−0.9	0.41	*trans*	*trans*	0.30/0.28	yes

ence of rotameric averaging about $\chi1$ for these residues was confirmed by intermediate $^3J_{CoCy}$ and $^3J_{NCy}$ coupling constants,[28] and is summarized in the final column of Table 1. The detection of dynamics was further compared to side chain model building, using the 1.6 Å X-ray crystal structure 4ICB[27] and the dominant conformations in the NMR family of structures 1B1G.[24] For the rigid side chains there is good agreement found with the conformations dictated by the ^{13}C chemical shifts. However, when dynamics is present, as gauged from ^{13}C chemical shifts, scalar couplings or relaxation-derived order parameters, discrepancies arise. For example, Leu6 is modelled in alternative conformations in the X-ray and NMR structures. More interestingly, in the room temperature crystal structure the Leu30 $\chi2$ angle was modelled *trans* and *gauche*+, with 2:1 occupancy. From the chemical shift data and Equation (2) we calculate $P_t=0.56$, indicating that both states are also highly populated in solution.

In summary, it is demonstrated here that the ^{13}C NMR γ-gauche effect gives rise to a pattern of leucine methyl chemical shifts that are sensitive to conformation and dynamics; this demonstrates the potential of chemical shift information as an intelligible source of protein structure and a quantitative measure of the extent of averaging around intervening dihedral angles. The temperature dependence of the methyl ^{13}C chemical shift difference provides additional information about the relative energies of the interconverting side-chain conformations. This information is the key to interpreting side chain dynamics from spin relaxation data, and may be a valuable restraint on side-chain conformation in ensemble or time-average structure refinement protocols.

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