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Leucine side-chain rotamers in a glycophorin A transmembrane peptide as revealed by three-bond carbon–carbon couplings and ^{13}C chemical shifts

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Summary

We have used a spin-echo difference NMR pulse sequence to measure three-bond J couplings between δ- and α-carbons of the leucine residues in a micelle-associated helical peptide dimer that corresponds to residues 62–101 of the transmembrane erythrocyte protein glycophorin A. The observed ^3J couplings correlate strongly with the ^{13}C chemical shift of the δ-methyl groups, and within experimental error both the shift distribution of the methyl carbons and the variations in ^3J can be accounted for by variations in side-chain rotamer populations. We infer that all leucine side chains in this peptide dimer are in fast exchange among χ^2 rotamers and sample two of the three possible rotameric states, even when the side chain forms part of the dimer interface. The observed correlation of chemical shift with couplings can be traced to a γ-gauche interaction of methyl and α-carbons. This correlation may provide an alternate route to rotamer analysis in some protein systems.

In the determination of a protein structure by solution NMR, experimentally derived restraints of backbone and side-chain torsion angles provide an important complement to the distance restraints afforded by the measurement of NOE cross-peaks between pairs of protons. Measured values for ^1H-^1H, ^1H-^{13}C, ^1H-^{15}N, ^{13}C-^{13}C and ^{13}C-^{15}N ^3J couplings in proteins have been used to identify secondary structure elements, to restrain side-chain torsion angles, and to assist in the stereospecific assignment of prochiral β-protons and γ-methyl carbons (Pardi et al., 1984; Montelione and Wagner, 1989; Wagner, 1990; Archer et al., 1991; Schmieder et al., 1991; Bax et al., 1992, Grzesiek et al., 1993; Vuister and Bax, 1993; Vuister et al., 1993a,b,1994; Rexroth et al., 1995). These J coupling data contribute significantly to the quality of the derived structural models.

Three-bond couplings also provide the possibility of directly measuring the extent of rotamer averaging that takes place around a given bond. The effective ^3J for two nuclei with a dihedral angle that is in fast exchange between different configurations is simply the sum of the couplings experienced in each configuration, weighted by the fraction of time spent in each state. Accurate determination of the effective coupling between two nuclei may permit the extraction of the rotamer population distribution. This approach has proven useful in small molecules (Booth, 1964), but has met with only limited success in proteins.

We are pursuing a structure determination of the helical, dimeric transmembrane domain of glycophorin A (GpA) in detergent micelles using heteronuclear solution NMR methods. Like many membrane-spanning sequences, the GpA transmembrane domain is rich in valine, leucine and isoleucine residues. While the positions of the γ-methyl groups of valine and isoleucine are sterically restricted to a single rotamer by helical backbone geometry, the δ-methyl groups of leucine and isoleucine do not experience such restrictions in isolated helices. Are these side chains rigid and located in specific, well-packed environments, or are they mobile, adding to the configurational entropy of the lipid solubilized transmembrane structures? Three-bond couplings involving the δ-methyl

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carbons can yield information on the χ^2 rotamers of these amino acids, but for hydrophobic peptides in detergent micelles such couplings can be extremely difficult to measure. Poor dispersion of the δ ^{1}H chemical shifts and of the C^{δ}, $C^{\gamma 2}$, and C^{α} ^{13}C shifts prevents the quantitative analysis of the experiment of Bax et al. (1992), which has been used to measure three-bond C^{δ}-C^{α} and C^{δ}-$C^{\gamma 2}$ couplings in soluble proteins; E.COSY-type experiments (Griesinger et al., 1986) are also adversely affected by the lack of chemical shift dispersion. However, two-dimensional constant-time (CT) spin-echo difference experiments for measuring ^{3}J couplings between γ-methyl carbons and backbone nitrogen (Vuister et al., 1993a) or carbonyl (Grzesiek et al., 1993) nuclei have been found to succeed for a number of the valine and threonine γ-methyl groups in our GpA sample. We show here that by applying a selective inversion pulse to the C^{α} spins of leucine residues, we can acquire a version of the CT spin-echo difference experiment that enables us to extend measurements to C^{δ}-C^{α} couplings. We also find a surprising correlation between the measured couplings and the chemical shifts of the δ-carbons of the leucine residues, which may prove useful in future work.

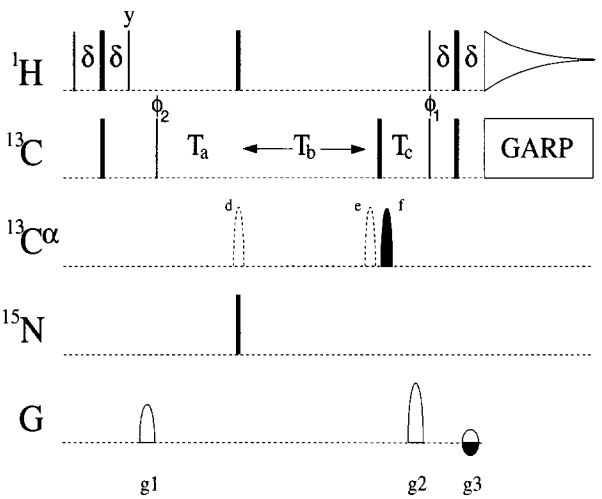

Fig. 1. Pulse sequence of the spin-echo difference experiment used to measure $^3J_{CC^{\alpha}}$ for leucine residues of the GpA peptide. Narrow (wide) lines represent 90° (180°) pulses. The values of delays T_a, T_b, and T_c were set such that $t_1 = T_a + T_b - T_c$ and the total time $T_a + T_b + T_c = 57.2$ ms. Delays δ were set to 1.8 ms. G3 inversion pulses on the C^{α} spins were applied by phase ramping of a 3.5-ms Gaussian cascade (Emsley and Bodenhausen, 1990) to the desired offset (56 ppm for leucine C^{α}) from the ^{13}C carrier (19 ppm). Reference and attenuated spectra were acquired with G3 pulses at positions d and e, respectively; all spectra were acquired with a G3 pulse at position f. Gradient selection of coherences was accomplished with sine-bell-shaped gradients of 1-ms duration and maximum strengths of g2 = 30 and g3 = 7.545 G/cm. Quadrature detection in t_1 was obtained using the switched gradient method and by adding and subtracting t_1 pairs in the time domain (Davis et al., 1992; Tolman et al., 1992). Gradient g1 was applied for 0.5 ms at a strength of 5 G/cm. Ringdown delays of 150 μs followed each gradient pulse. GARP decoupling (Shaka et al., 1985) was applied during the acquisition period with a B_1 field strength of 4 kHz. All pulses were of phase x, unless otherwise noted. Phase cycling was: ϕ_1: x,–x; receiver: x,–x. The phase of pulse f was cycled (x,x,–x,–x) without cycling the receiver. The acquisition of t_1 points of the reference and attenuated spectra was interleaved.

The concept of utilizing the spin-echo difference method within a constant-time HSQC to measure small couplings was first described by Vuister et al. (1993a). Two spectra are acquired: a reference 2D CT-HSQC spectrum with the coupling of interest refocussed during the indirect evolution period, and an attenuated 2D CT-HSQC spectrum with the coupling of interest active during the indirect evolution period. The ratio of the integrated intensities in the reference and difference (reference minus attenuated) spectra can be related to the magnitudes of the couplings. The pulse sequence used to measure the C^{δ}-C^{α} couplings, as diagrammed in Fig. 1, consists of a 2D constant-time ^{1}H-^{13}C HSQC in which a C^{α}-selective inversion pulse is applied at position d to acquire a reference spectrum and at position e to acquire an attenuated spectrum. Because the hard π pulse in the t_1 period is not intended to invert the C^{α} spins, in both reference and difference spectra a C^{α}-selective π pulse is applied at position f. The C^{α}-selective pulses should not perturb the C^{δ} spins or the other coupling partners of C^{δ} (i.e., C^{β} and C^{γ}); the G3 selective inversion pulse (Emsley and Bodenhausen, 1990) is well suited to this purpose.

Errors that affect the quantitative performance of this type of pulse sequence have been described by Grzesiek et al. (1993). Off-resonance perturbations by the G3 pulses were estimated by examining the alanine β-methyl intensities and varying the frequency to which the G3 pulse is phase-ramped. Because of the constant time period used and the strong J coupling between C^{α} and C^{β} (36 Hz), this test of the pulse sequence enables us to conclude that off-resonance effects of the G3 pulse attenuate the measured couplings by less than 3%. The 4.6 kHz offset of the C^{α} spins from the carrier resulted in less than 2% attenuation of the measured couplings due to incomplete inversion by the hard ^{13}C π pulse in t_1; this could have been reduced by placing the carrier halfway between the C^{α} and C^{δ} resonances. Following the arguments of Grzesiek et al. (1993), we expect that the calculated couplings underestimate the true couplings by no more than 10%; the reported couplings are uncorrected.

The leucine δ-methyl region of the reference spectrum is shown in Fig. 2A. The resolution available in the ^{13}C shift dimension alleviates the poor chemical shift dispersion in the ^{1}H dimension and permits all but two of the methyl groups to be resolved from one another. The intensities in both the spin-echo difference spectrum presented in Fig. 2B and the reference spectrum were quantitated, and the calculated couplings and statistical errors are presented in Table 1. The excellent signal-to-noise ratio of the difference spectrum is reflected in the low statistical errors associated with the calculated $^3J_{CC^{\alpha}}$ for most of the leucine methyl groups.

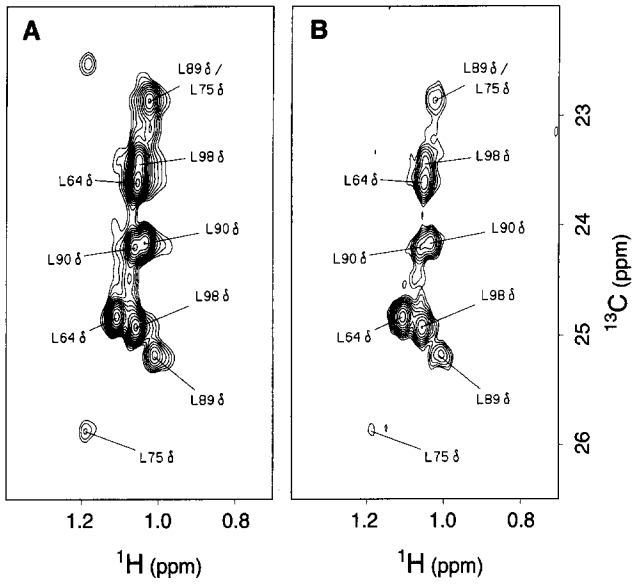

Fig. 2. Reference and spin-echo difference spectra used to measure $^3J_{CC^\alpha}$ for leucine methyls of the GpA peptide. Spectra were acquired at 40 °C using a GE Ω500 spectrometer with waveform capabilities on all three rf channels, utilizing a Bruker triple-resonance (^{1}H,^{15}N,^{13}C) triple-axis-pulsed field gradient probe. The sample consists of a uniformly (^{15}N,^{13}C)-labeled peptide corresponding to residues 62–101 of human glycophorin A, solubilized at a monomer concentration of 3.5 mM in 200 mM d_{38}-dodecylphosphocholine and water containing 10% D_2O. The ^{1}H, ^{15}N and ^{13}C carriers were set on the H_2O resonance, at 116 ppm and at 19 ppm, respectively. Dwell times in both the direct and indirect dimensions were 200 μs. For reference and attenuated spectra, 256 complex points each were acquired in t_1, and 512 complex points were acquired in t_2; the total acquisition time was 10 h. Spectra were processed in FELIX v. 2.3 (BioSym Technologies, San Diego, CA) using a cosine bell in the direct dimension and a Kaiser window in t_1. Final matrix dimensions were 4096 by 4096 real points.

All measured values of $^3J_{CC^\alpha}$ (1.8–3.5 Hz) fall between the values reported for gauche (0.9 Hz) and trans (4.0 Hz) three-bond carbon–carbon J couplings in small aliphatic molecules and in proteins (Bystrov, 1976; Krivdin and Della, 1991; Bax et al., 1992; Grzesiek et al., 1993; Vuister et al., 1993b). Two mechanisms may be proposed to account for these intermediate couplings: the methyls may be in exchange between trans and gauche configurations, or they may have torsion angles that do not correspond to either of these rotamer positions. Arguments can be made for rotamer averaging and against a model that invokes nonideal torsions in the case of the leucine residues, where two C^δ-C^α couplings can be measured for each side chain. The observed pairs of couplings do not correspond to nonideal torsions separated by 120° when compared to the Karplus relation for ^{13}C-^{13}C couplings (Krivdin and Della, 1991), but they are consistent with reasonable population fractions of the two singly gauche χ^2 rotamers of leucine. Table 1 shows the C^δ-C^α trans rotamer fraction for each methyl carbon, calculated using the coupling data and the $^3J_{CC}$ empirical values for trans and gauche configurations in small molecules.

In addition, it turns out that evidence for rotamer averaging is present in the ^{13}C chemical shifts of the leucine δ-methyl groups. Both chemical shift and J coupling would be time-averaged under conditions of rapid rotamer averaging. ^{13}C chemical shifts in small molecules are known to be strongly affected by steric interactions between vicinal carbons: gauche configurations experience a 6.4 ppm upfield shift relative to trans configurations (Dalling and Grant, 1967). The striking correlation of $^3J_{CC^\alpha}$ and the leucine methyl ^{13}C chemical shift in Fig. 3 suggests that both measurables are dependent on the same physical parameter, and the slope of the least-squares fit line to these points is 0.51 Hz/ppm. This corresponds closely to the predicted slope (0.48 Hz/ppm) for a rotamer averaging model in which the small-molecule J and shift parameters are assigned to the trans (J = 4.0 Hz, $\Delta\sigma = 0$ ppm) and gauche (J = 0.9 Hz, $\Delta\sigma = -6.4$ ppm) methyl carbons. Together, the coupling and shift data provide compelling evidence that the leucine methyls of the GpA transmembrane domain are rotamer-averaged between conformers that correspond to the energetic minima observed in small molecules.

Two leucine δ-methyl groups, belonging to residues Leu^{75} and Leu^{89}, are not included in the linear fit of Fig. 3 because their overlap prevents accurate determination of $^3J_{CC^\alpha}$. However, the rotamer averaging model described above can be applied to predict the couplings and chemical shifts of these unresolved methyls using the population fractions calculated for the resolved methyls. Assuming that the (gauche,gauche) conformer is negligibly populated, and propagating the calculated population fraction errors from Table 1, we predict ^{13}C chemical shifts for the overlapped methyls of Leu^{75} and Leu^{89} of 23.3 ± 0.4 ppm and 23.5 ± 0.2 ppm, respectively. The experimental shift of 22.9 ppm is quite close to these predicted values. The predicted $^3J_{CC^\alpha}$ values for the overlapped

TABLE 1
^{13}C SHIFTS, $^3J_{CC^\alpha}$ VALUES AND C^δ-C^α *trans*-ROTAMER FRACTIONS FOR RESOLVED LEUCINE δ-METHYL CARBONS

Leucine $^{13}C^\delta$ resonance	^{13}C shift (ppm)	J_{CC^α} (Hz)	$\phi_{trans}(C^\alpha)$
Leu^{64}	23.62	2.0 ± 0.1	0.35 ± 0.03
	24.83	2.8 ± 0.1	0.61 ± 0.03
Leu^{75}	25.90	3.0 ± 0.2	0.68 ± 0.06
Leu^{89}	25.20	2.9 ± 0.1	0.65 ± 0.03
Leu^{90}	24.17	2.4 ± 0.2	0.48 ± 0.06
	24.20	2.2 ± 0.2	0.48 ± 0.06
Leu^{98}	23.47	1.8 ± 0.1	0.29 ± 0.03
	24.93	2.7 ± 0.1	0.55 ± 0.03

Couplings were calculated from the measured intensities (S_{ref} and S_{diff}) and noise levels (ε) in the 2D spectra using the following equation: $J_{CC^\alpha} = (1/\pi T)\sin^{-1}((S_{diff} \pm \varepsilon)/S_{ref})^{1/2}$. The time T has been corrected for the decoupling effect of the two G3 selective pulses (Grzesiek et al., 1993).

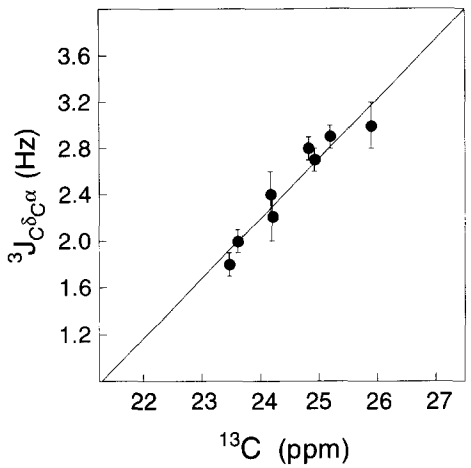

Fig. 3. Correlation of leucine δ-carbon chemical shifts and C^{δ}-C^{α} J couplings for residues of the GpA peptide. Uncorrected coupling data are plotted for the eight resolved leucine methyl peaks of transmembrane GpA. Error bars express the statistical error in $^3J_{CC^{\alpha}}$, and do not include any estimate of systematic error. The correlation coefficient R for the least-squares linear fit to the data points is 0.96; the slope of the line is 0.51 Hz/ppm.

methyls of Leu^{75} and Leu^{89} are 1.7 ± 0.2 Hz and 2.0 ± 0.1 Hz; this is quite close to the experimental combined difference intensity, which corresponds to a coupling of 1.7 ± 0.2 Hz.

The excellent correlation observed in Fig. 3 and the agreement with small-molecule empirical observations encouraged us to search for this correlation in the protein NMR literature. A correlation is seen between $^3J_{CC^{\alpha}}$ and methyl ^{13}C chemical shift in Fig. 4 for data from staphylococcal nuclease, but the scatter is substantial. Although there seems to be some generality to the J coupling/chemical shift correlation in proteins, either local environmental factors or deviations from ideal bond and torsion angles affect the chemical shifts and/or couplings sufficiently to weaken the correlation to the point that the shift data cannot be reliably used to predict rotamer populations.

It is noteworthy that the range of couplings reported for the leucines of GpA corresponds to a population fraction regime where the two singly gauche χ^2 rotamers are preferred only slightly over one another, and the doubly gauche rotamer is marginally populated. While it may seem unremarkable that lipid- or detergent-exposed residues would average between the (sterically available) small-molecule energetic minima, the fact that the methyls of Leu^{75}, which has been shown by mutagenesis to be critical to the dimerization of GpA, are also part of this correlation is more intriguing. Despite the exquisite sequence dependence of glycophorin A dimerization on the residue type at this site (valine completely and isoleucine almost completely abrogates dimerization; Lemmon et al., 1992), the terminal methyls appear to favor one configuration over another by a factor of perhaps no more than 2:1. As the calculated χ^2 rotamer populations are quite similar to those observed for leucines involved in less specific interactions with the detergent micelle, the involvement in and specificity of Leu^{75} in the dimer interface is found to be compatible with a distribution of rotamers similar to that expected for a monomeric GpA molecule.

This observation is in marked contrast with the reported data for the leucine methyls of staphylococcal nuclease. Of the 10 leucines in nuclease for which C^{δ}-C^{α} couplings have been reported for both methyls, three have pairs of methyl groups whose $^3J_{CC^{\alpha}}$ values cannot be distinguished from the all-trans and all-gauche small-molecule data (Vuister et al., 1993b). Two more residues have one coupling greater than 3.5 Hz and one coupling lower than 1.8 Hz, which are the upper and lower limits observed for the GpA couplings. Only three leucines show intermediate values of $^3J_{CC^{\alpha}}$ that are interpreted, on the basis of other coupling and relaxation data, as evidence of rotamer averaging (Nicholson et al., 1992; Vuister et al., 1993b). Thus, while some leucines in staphylococcal nuclease undergo rotamer averaging, most are considerably more ordered than Leu^{75} of GpA.

This comparison lacks the sampling needed to make generalizations about the differences between the hydrophobic cores of soluble proteins and helix–helix interfaces in hydrophobic transmembrane oligomers, but it does highlight the potential for these types of measurements to assist in the elucidation of the detailed structural properties of individual residues composed of amenable spin systems. The applicability of our pulse sequence to systems where the methyl ^1H chemical shift dispersion is poor opens the possibility of obtaining such coupling data

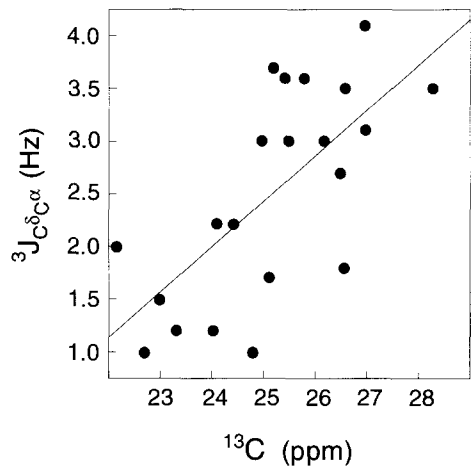

Fig. 4. Correlation of leucine δ-carbon chemical shifts and C^{δ}-C^{α} J couplings for residues of Staphylococcal nuclease. The shift and J coupling data reported by Vuister et al. (1993b) are plotted for the leucine methyls of staphylococcal nuclease ligated with thymidine 3',5'-bisphosphate and Ca^{2+}. The correlation coefficient R for the least-squares linear fit to the data points is 0.69; the slope of the line is 0.43 Hz/ppm.

from other membrane peptides and from partially folded or 'molten globule' proteins to determine the extent of side-chain order at points along a folding pathway. The correlation of leucine δ-methyl ^{13}C chemical shift with $^{3}J_{CC^{\alpha}}$ as seen in the GpA system suggests that even slight differences in the extent of averaging should result in chemical shift differences, which will help reduce overlap problems, as well as in coupling differences that should prove interpretable in terms of simple rotamer averaging models. These measurements should provide information complementary to the ^{1}H-^{1}H NOE-derived distance restraints, $^{3}J_{HN\text{-}H^{\alpha}}$ couplings, and relaxation studies (Neri et al., 1992; Shortle and Abeygunawardana, 1993; Alexandrescu et al., 1994; Logan et al., 1994; Farrow et al., 1995; Frank et al., 1995) that have so far been used to characterize the conformational state of unfolded proteins.

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