Accurate Orientation of the Functional Groups of Asparagine and Glutamine Side Chains Using One- and Two-Bond Dipolar Couplings

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Received June 19, 2001
Revised Manuscript Received July 16, 2001

Residual dipolar couplings measured in a dilute liquid crystalline phase[1] provide unique long-range orientational information that has been shown to lead to substantial improvements in backbone accuracy for NMR structures of proteins[2] and protein−protein complexes.[3] Much of the focus has been on backbone dipolar couplings. Recently, it has been shown that χ_1 rotamers can be identified from analysis of $^1D_{C\beta H\beta}$, $^1D_{C\alpha N}$, $^1D_{C'C\alpha}$, and $^1D_{C\alpha H\alpha}$ couplings.[4] In this paper, we present a simple approach based on dipolar couplings for obtaining accurate orientations of the carboxyamide functional group of Asn (χ_2) and Gln(χ_3) side chains. These are of considerable interest since the functional groups of Asn and Gln are often involved in specific hydrogen bonding interactions.

There are five easily measured dipolar couplings that can be used to determine the orientation of the carboxyamide group of Asn and Gln in an NMR-based protein structure refinement: namely, $^1D_{N\delta2-H\delta21}$, $^1D_{N\delta2-H\delta22}$, $^1D_{N\delta2-C\gamma}$, $^2D_{C\gamma-H\delta21}$, and $^2D_{C\gamma-H\delta22}$ for Asn, and $^1D_{N\epsilon2-H\epsilon21}$, $^1D_{N\epsilon2-H\epsilon22}$, $^1D_{N\epsilon2-C\delta}$, $^2D_{C\delta-H\epsilon21}$, and $^2D_{C\delta-H\epsilon22}$ for Gln. To make use of these couplings, it is first necessary to stereospecifically assign the side chain NH_2 protons of Asn and Gln. Methods based on three-bond scalar couplings between the NH_2 protons and Cβ atom for Asn and Cγ atom for Gln have been described.[5] These experiments, however, are relatively time-consuming. We demonstrate a different approach using two-bond heteronuclear scalar ($^2J_{HNC}$) couplings between the NH_2 protons and side chain carbonyl carbon atoms (Cγ atom for Asn and Cδ for Gln). The Hδ21 (Asn) and Hϵ21 (Gln) protons are trans to the side-chain oxygen (Oδ1 and Oϵ1, respectively) of the carboxyamide group, analogous to the relationship between the backbone amide proton and carbonyl oxygen in a trans peptide bond, and have a positive $^2J_{HNC}$ coupling of 2.5−5.0 Hz; the Hδ22 (Asn) and Hϵ22 (Gln), on the other hand, are cis to the side chain oxygen, and have a negative $^2J_{HNC}$ coupling of −2.5 to −5.0 Hz.[6] These couplings are readily measured from a carbonyl-coupled $n = 2$ multiplicity-edited 2D $^1H-^{15}N$ heteronuclear quantum coherence (HSQC) correlation spectrum in which only cross-peaks for the side chain NH_2 groups are observed[7] (Figure 1a). The basic pulse sequence (fully decoupled version) employed is given in Figure 1a, and the F_1-carbonyl coupled spectrum for the 168 residue protein LAP2, whose structure has recently been solved,[9]

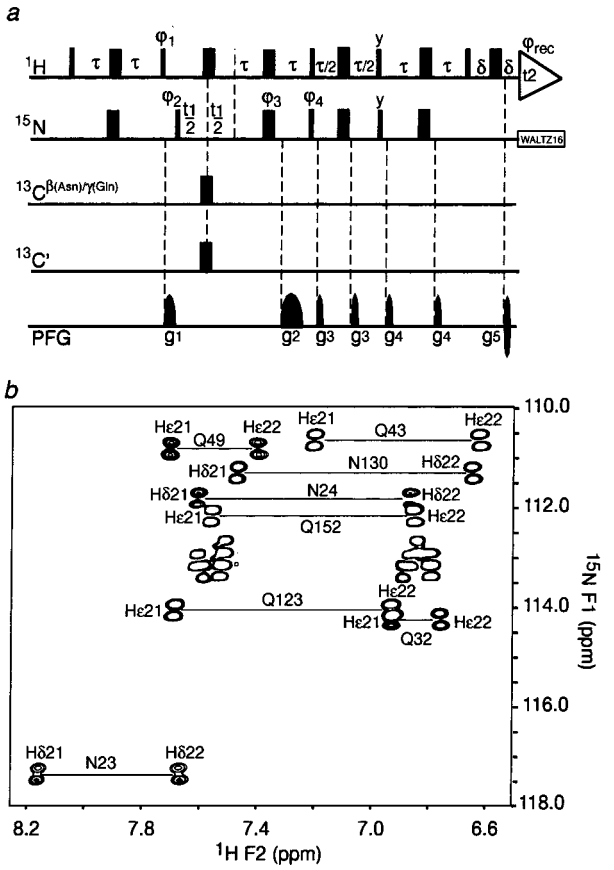

Figure 1. Stereospecific assignment and measurement of residual dipolar couplings for the side chain amide groups of Asn and Gln. (a) The basic fully decoupled, gradient-based, $n = 2$ multiplicity-edited[7] $^1H-^{15}N$ HSQC experiment designed to selectively observe NH_2 correlations. Narrow and wide pulses correspond to flip angles of 90° and 180°, respectively. The length (τ_{180}) of the 180° $^{13}C\beta/\gamma$ and ^{13}C carbonyl pulses is chosen such that they have a null at the positions of the ^{13}C carbonyl and $^{13}C\beta/\gamma$ resonances, respectively ($\tau_{180} = \sqrt{3}/2\Delta$, where Δ is the frequency difference between the $^{13}C\beta/\gamma$ and ^{13}C carbonyl resonances). The delay τ is set to $^1/_4J_{NH} = 2.6$ ms. The delay δ has a value of 400 μs. All pulse phases are x, unless otherwise specified. Phase cycling: $\varphi_1 = 2(y)$, $2(-y)$; $\varphi_2 = x, -x$; $\varphi_3 = 4(x)$, $4(y)$, $4(-x)$, $4(-y)$; and receiver phase = x, $2(-x)$, x, $-x$, $2(x)$, $-x$. Rance−Kay t_1 quadrature detection is used,[7] alternating the phase of φ_4 between x and $-x$ in concert with the polarity of the pulsed field gradient (PFG) g5. All PFGs are sine-bell shaped (30 G/cm except g4 which is 21 G/cm). The durations of g1, g2, g3, g4, and g5 are 1.5, 2.0, 0.3, 0.3, and 0.203 ms, respectively. For stereospecific assignments of the Hδ21 and Hδ22 hydrogens of Asn and the Hϵ21 and Hϵ22 hydrogens of Gln, and for the measurement of side chain $^1D_{NC}$ and $^2D_{HNC}$ dipolar couplings, the 180° carbonyl pulse during the evolution period t_1 is omitted; for measurement of $^1D_{NH}$ side chain dipolar couplings, no broad-band decoupling is employed during acquisition. (b) 2D F_1-carbonyl coupled multiplicity-edited $^1H-^{15}N$ HSQC spectrum of LAP2 collected in isotropic medium (water) using the pulse sequence shown in (a) omitting the carbonyl 180° pulse during t_1.

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10.1021/ja0164475
Published on Web 08/10/2001

is shown in Figure 1b. The $^2J_{HNC}$ couplings are measured from the displacement of the upper component of the multiplet relative to the lower one in the ^1H (F_2) dimension of the spectrum with positive and negative slopes indicative of positive and negative $^2J_{HNC}$ couplings, respectively (Figure 1b). In this particular instance all the Hδ21 and Hϵ21 protons of Asn and Gln are downfield from their respective Hδ22 and Hϵ22 partners (Figure 1b).

The five dipolar couplings involving the side chain amides of Asn and Gln in LAP2 were measured in a liquid crystalline medium of phage pf1[1c] (15 mg/mL). The side-chain $^1D_{NC}$ and $^2D_{HNC}$ couplings are determined from the splittings in the F_1 and F_2 dimensions, respectively, of the F_1-carbonyl coupled version of the experiment shown in Figure 1a. The side-chain $^1D_{NH}$ couplings are measured in the t_2 acquisition dimension from the $F_2-^{15}N$ coupled/$^{13}C'$-decoupled version of the experiment.[10]

The presence of significant motion about χ_2 for Asn and χ_3 for Gln was assessed by recording a steady-state $^{15}N-\{^1H\}$ NOE experiment:[11] the side-chain amide groups of Asn23, Gln49, Gln32, and Gln132 have $^{15}N-\{^1H\}$ NOE values of 0.87, 0.65, 0.50, and 0.30, respectively; all other side-chain amide groups had $^{15}N-\{^1H\}$ NOE values that were either close to zero or negative. Thus, the side-chain amide of Asn23 is essentially as rigid as the backbone, while the side-chain amide groups of the other Asn and Gln display varying degrees of internal motion.

We therefore chose to use the data on Asn23 to assess the usefulness and impact of side-chain dipolar couplings on the refinement of the positions of the carboxyamide groups of Asn and Gln. Structures were calculated in torsion angle space by simulated annealing by using the NIH version of XPLOR[12] with and without the side-chain dipolar couplings of Asn23 included in the calculations.[13] All other experimental restraints were identical in the two sets of calculations.[9,13] Included in these restraints were a χ_1 torsion angle restraint ($-60 \pm 20°$) for Asn23 (previously determined from a 30 ms mixing time 3D ^{13}C-separated NOE spectrum recorded in H_2O[9]) and a weak NOE-derived interproton distance restraint from the side-chain amide protons of Asn23 to the CαH proton of Glu19. The results are displayed in Figure 2, which shows a best-fit superposition of 20 simulated annealing structures for the main chain atoms of residues 18−25 and the side-chain atoms of Asn23. In the absence of side-chain dipolar couplings for Asn23 (Figure 2a), the orientation of the carboxyamide group falls into two clusters with χ_2 angles of $-42 \pm 3°$ and $167 \pm 15°$. The value of the dipolar coupling *R*-factor[14] (R_{dip}^{Asn23}) for the 5 measured dipolar couplings of the Asn23 side chain is $54 \pm 5\%$ for the first cluster and $106 \pm 17\%$ for the second cluster. (In both cases, the χ_1 angle is very close to $-60°$.) In the first cluster, the NH_2 group of Asn23 could form a potential hydrogen bond with the main chain carbonyl oxygen atom of Glu19 (Figure 2a). While the Nδ2(23)−O(19) distance of 2.64 Å is reasonable for such a hydrogen bond, the Hδ21 and Hδ22 atoms of Asn23 are almost equidistant from the carbonyl oxygen of Glu19 (2.74 and 3.07 Å, respectively) and the Nδ2(23)−Hδ21(23)−O(19) angle has a value of $84 \pm 2°$. Thus, the hydrogen bond geometry for the first cluster is very poor. For the second cluster, the amide group of Asn23 is too far away and in an inappropriate orientation to form a hydrogen bond with the backbone of Glu19 (Figure 2a). When the side chain dipolar couplings of Asn23, however, are included in the calculation (Figure 2b), R_{dip}^{Asn23} is reduced to $22 \pm 1\%$ and all simulated annealing structures display the same side chain orientation of Asn23 with a χ_2 angle of $-70 \pm 3°$. The distances from the Nδ2 and Hδ21 atoms of Asn23 to the backbone carbonyl oxygen of Glu19 are 2.82 ± 0.10 and 2.05 ± 0.09 Å, respectively, and the Nδ2(23)−Hδ21(23)−O(19) angle is $135 \pm 4°$, fully consistent with good stereochemistry for a hydrogen bond between the Hδ21 atom of Asn23 and the backbone carbonyl oxygen of Glu19.

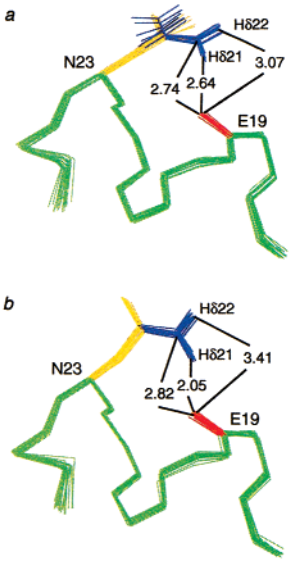

Figure 2. Impact of side chain dipolar couplings on the orientation of the carboxyamide group of Asn23 of LAP2. Best-fit superposition of the main chain of residues 18−25 and the side chain of Asn23 of 20 simulated annealing structures calculated (a) without and (b) with $^1D_{N\delta21-H\delta21}$, $^1D_{N\delta2-H\delta22}$, $^1D_{N\delta2-C\gamma}$, $^2D_{H\delta22-C\gamma}$, and $^2D_{H\delta22-C\gamma}$ dipolar couplings for Asn23. The main chain atoms are shown in green and the backbone carbonyl (C′−O) of Glu19 in red; the side chain amide of Asn23 is in blue and the remainder of the Asn23 side chain in yellow. The distances in Å from the Nδ2, Hδ21, and Hδ22 atoms of Asn23 to the backbone carbonyl oxygen atom of Glu19 are indicated.

We have shown that side-chain dipolar couplings involving the carboxyamide group of Asn and Gln are readily measured and can have a significant impact on the accuracy with which the orientation of the functional group of Asn and Gln can be determined by NMR, thereby shedding light on and improving the geometry of specific hydrogen bonding interactions.

Acknowledgment. This work was supported by the AIDS Targeted Antiviral Program of the Office of the Director of the National Institutes of Health (to G.M.C.).

JA0164475

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(10) Although the accuracy of the $^1D_{NH}$ couplings can potentially be affected by cross-correlations between H−H and N−H dipolar couplings, this effect is generally small, and its magnitude can be assessed by processing the spectrum with varying degrees of line broadening in the t_2 dimension.

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(13) LAP2 comprises two domains (residues 1−50 and 111−152) connected by a highly flexible 60 residue linker.[9] Since the two domains reorient independently of each other, calculations for the present work were restricted to the first domain for which there are 769 experimental NMR restraints (excluding the 5 dipolar couplings for the side chain of Asn23). These comprise 395 interproton distances, 151 torsion angles, 105 ^{13}C shifts, and 123 backbone dipolar coupling restraints. The values of the axial component of the alignment tensor (D_a^{NH}, normalized to the N−H bond vectors) and the rhombicity are −7.2 Hz and 0.62, respectively. The force constants employed for the normalized $^1D_{NH}$, $^1D_{NC}$, and $^2D_{HNC}$ dipolar couplings are 1.0, 0.05, and 0.308 kcal·mol^{-1}·Hz^{-2}, respectively.

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