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The HN(COCA)HAHB NMR Experiment for the Stereospecific Assignment of H_β-Protons in Non-native States of Proteins

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The characterization of conformational dynamics observed in non-native states of proteins is of particular importance to further our understanding of protein folding and misfolding. As a class of non-native protein states, intrinsically unstructured proteins (IUPs) have gained particular interest lately due to their important role in protein folding diseases.[1] NMR spectroscopy is the technique of choice to investigate the dynamic ensemble of conformational states characterizing non-native states of proteins. Previous NMR spectroscopic investigations based on *J*-coupling analysis as well as RDC measurement have revealed significant differences in amino acid specific sampling of ϕ,ψ space, but little is known about conformational preferences characterizing the side-chain angle χ_1. However, from the analysis of several homo- and heteronuclear ^3J-couplings the distribution of the χ_1 side-chain torsion angle can be determined.[2] Of the six possible couplings defining χ_1, the $^3J(H_\alpha,H_\beta)$-coupling constant is the best parametrized and has been measured for small unstructured peptides,[3] but not for larger polypeptide chains due to the limited spectral resolution.

We report here the development of a novel HN(COCA)HAHB experiment for the determination of these $^3J(H_\alpha,H_\beta)$-coupling constant in non-native states of proteins. In these states, the 2D H,N-correlation spectrum is the only well-resolved spectral region, and the proposed experiment exploits this resolution. The desired coupling is extracted from the intensity ratios of two peaks, generated by an in-phase COSY transfer of magnetization (Figure 1) following the quantitative *J*-correlation approach.[4−6] The experiment consists of 4 INEPT steps to transfer magnetization from H^N via N,C′ and C_α to H_α and a COSY-like magnetization transfer between H_α and H_β, encoding the size of the coupling as the ratio of cross-to-diagonal peak intensity. The backtransfer is symmetric with a constant time evolution on N and a watergate sequence for solvent suppression (Figure 2). The $^3J(H_\alpha,H_\beta)$-coupling constant is extracted from the ratio of signal volumes or intensities (in case of limited resolution) between H^N-N-H_β cross and H^N-N-H_α diagonal peak according to:

$$S_{CP}/S_{DP} = -(\tan)^2(\pi \times {}^3J(H_\alpha, H_\beta) \times 2\zeta)$$

In the new experiment, also an $H_\alpha-H^N$ correlation is generated, and the intensity of the cross peak depends on the $^3J(H^N,H_\alpha)$-coupling constants. We use this information to independently cross-validate our novel data. The $^3J(H^N,H_\alpha)$-coupling constants were found to be within a range of ±5% identical to those obtained independently in an HNHA reference experiment.[7]

In order to arrive at a stereospecific assignment of the two diastereotopic protons, two additional heteronuclear coupling constants, namely $^3J(N,C_\gamma)$ and $^3J(C',C_\gamma)$ measured in HNCG-[8,9] and HN(CO)C-[10] experiments, respectively, were used as input for a Pachler-type analysis.[11,12] Such analysis assumes that the χ_1 angle distribution can be modeled by the three staggered conformers; the

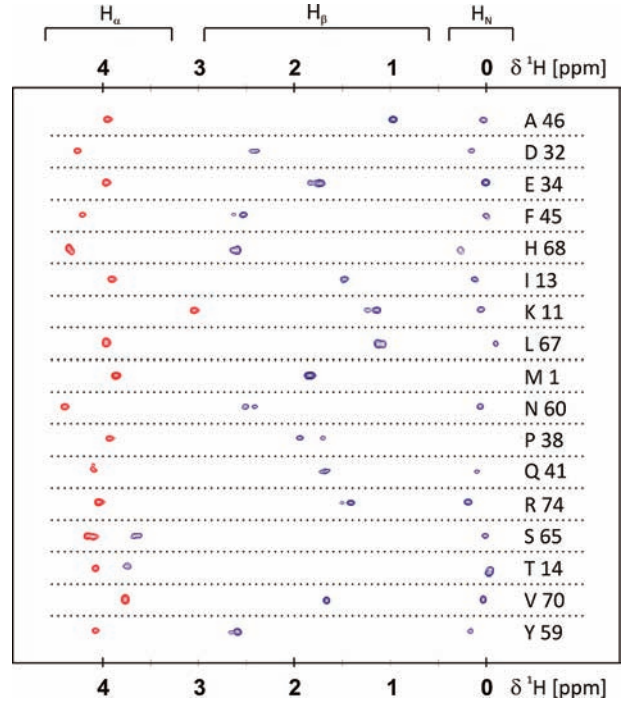

Figure 1. Examples of 1D strip plots of the ω_1 dimension containing the H_β and folded H^N crosspeaks as well as H_α diagonal peaks, taken from the 3D HN(COCA)HAHB spectrum at the $^1H^N$ and ^{15}N chemical shifts of the corresponding $(n + 1)$th amino acid. Experiments were recorded on a Bruker AV700 spectrometer equipped with an 5 mm TXI ^1H $\{^{13}C/^{15}N\}$ triple resonance cryoprobe with *z*-gradient coils. A total of 172 × 72 × 1024 data points (ω_1: $H_{\alpha/\beta}$, ω_2: N, ω_3: H^N) corresponding to acquisition times of 25, 23, and 53 ms, respectively, were recorded over a time of 17 h with spectral widths of 5, 22, and 14 ppm, respectively, and four accumulated scans per transient. Spectra were recorded on a sample containing 1.5 mM ubiquitin unfolded in 8 M urea at a pH of 2 and 298 K, using the resonance assignment by Peti et al.[13]

observed averaged coupling constants can then be described by the population-weighted couplings:

$$\langle J_{obs} \rangle = p_{180^\circ} \times J_{180^\circ} + p_{60^\circ} \times J_{60^\circ} + p_{-60^\circ} \times J_{-60^\circ}$$

with p_x and J_x being the population and the expected coupling for each state. The population of an $N-C_\alpha-C_\beta-C_\gamma$ angle of 180°, corresponding to $\chi_1 = 180°$ (in non β-branched amino acids), can be calculated from $^3J(N,C_\gamma)$ and the population of $C'-C_\alpha-C_\beta-C_\gamma = 180°$ corresponding to $\chi_1 = -60°$ from $^3J(C',C_\gamma)$.

An inverse Pachler analysis based on χ_1-populations and $^3J(H_\alpha,H_\beta)$-couplings for *trans*- and *gauche*-conformations published by Schmidt[14] was performed to calculate population-weighted couplings. These showed to be in good agreement with $^3J(H_\alpha,H_\beta)$-couplings acquired experimentally and lead to a stereospecific

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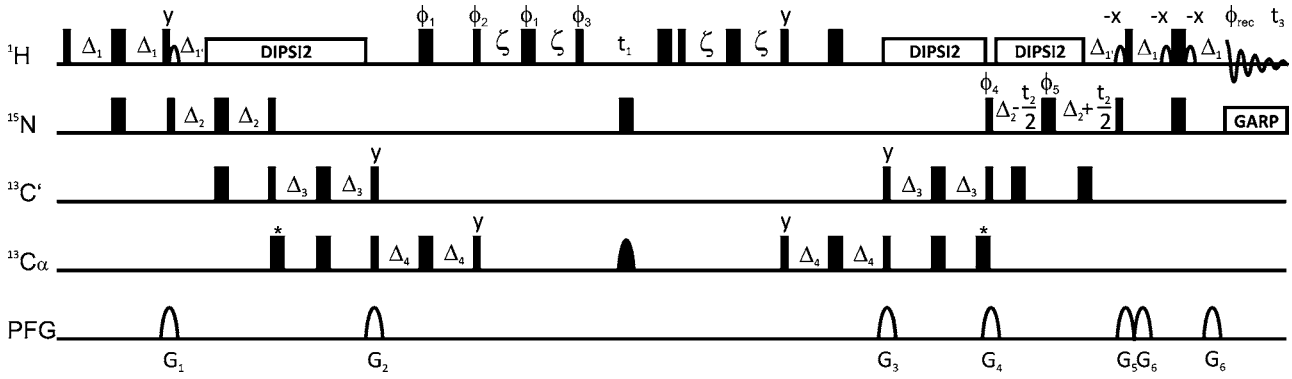

Figure 2. Pulse scheme of the HN(COCA)HAHB experiment. Narrow and wide pulses correspond to flip angles of 90° and 180°, respectively. All pulses are applied along the *x*-axis unless indicated otherwise. The phase cycle used is: $\phi_1 = x$; $\phi_2 = y$; $\phi_3 = x, -x$; $\phi_4 = 2x, 2(-x)$; $\phi_5 = 4x, 4(-x)$; $\phi_{rec} = 2x, 2(-x)$. The delay durations are: Δ_1 ($\approx 1/4 \times {}^3J(H^N,N)$) = 2.3 ms, $\Delta_{1'}$ ($\approx 1/2 \times {}^3J(H^N,N)$) = 5.5 ms, Δ_2 ($\approx 1/4 \times {}^1J(N,C')$) = 12 ms, Δ_3 ($\approx 1/4 \times {}^1J(C',C_\alpha)$) = 4 ms, Δ_4 ($\approx 1/4 \times {}^1J(C_\alpha,H_\alpha)$) = 1.5 ms, $\zeta = 10$ ms. Carrier frequency positions are 4.7 ppm for H^N (switched to 3 ppm for H_α and H_β between G_2 and G_3), 117 ppm for N, 54 ppm for C_α, and 176 ppm for C′. Pulses on ^{1}H and ^{15}N are rectangular hard pulses with an applied RF field strength of 17.5 kHz and 6.5 kHz, respectively, whereas pulses on C′ and C_α are selective shaped Gaussian cascades except for the central adiabatic CHIRP pulse (affecting C′ carbons as well as C_α carbons) marked as sinoidal pulse shape. The applied pulsed field gradients have a sine-bell shape with a strength of $G_1 = 27.5$ G/cm, $G_2 = 44$ G/cm, $G_3 = 38.5$ G/cm, $G_4 = 22$ G/cm, $G_5 = 33$ G/cm, $G_6 = 16.5$ G/cm and a length of 1 ms. DIPSI2-decoupling is applied at 4.7 ppm using a field strength of 3.1 kHz. Heteronuclear scalar couplings during acquisition are suppressed by asynchronous GARP decoupling on the nitrogen channel at an RF field strength of 0.83 kHz. Quadrature detection in t_1 and t_2 is obtained with the States-TPPI method, incrementing phases ϕ_1, ϕ_2 and ϕ_3 for the t_1-dimension and ϕ_4 for the t_2-dimension. Pulses for Bloch-Siegert suppression are indicated by an asterisk.

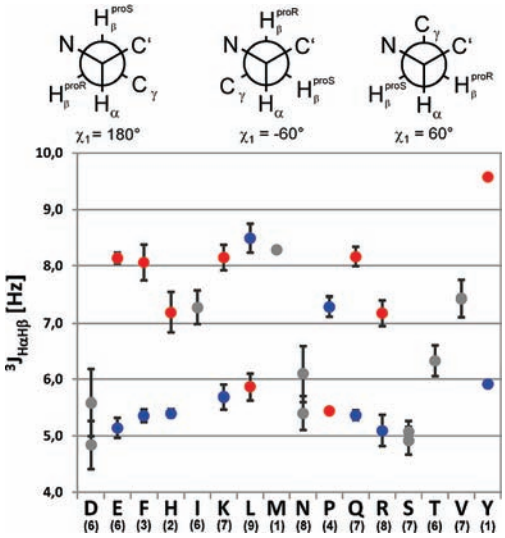

Figure 3. Staggered χ_1 rotamers and average $^3J(H_\alpha H_\beta)$-coupling constants in unfolded ubiquitin and an unfolded lysozyme mutant (C→A) by amino acid type. The number of examined amino acids is shown in parentheses. The following coupling constants were found for alanine residues: ubiquitin: 6.7 ± 0.2 Hz, lysozyme: 6.0 ± 0.2 Hz. Relative peak positions are color coded (red = downfield, blue = upfield).

assignment of H_β resonances. This approach is limited by the small number of complete $^3J(N,C_\gamma)$ and $^3J(C',C_\gamma)$ data sets caused by the small spectral dispersion in the carbon dimension of intrinsically unfolded proteins, which leads for ubiquitin to 24 stereospecific assigments although full $^3J(H_\alpha,H_\beta)$-data sets for 31 of the 42 observable residues with two β protons were determined.

In summary, we have developed a novel pulse sequence for measuring the size of the $^3J(H_\alpha,H_\beta)$-coupling constants in unfolded proteins, providing a simple method for stereospecific assignment of H_β^{proR} and H_β^{proS} resonances.

Supporting Information Available: Detailed information on the measured $^3J(H_\alpha,H_\beta)$-couplings, exemplary 1D slices for each amino acid type, the expected couplings for *trans*- and *gauche*-conformations, the full data set of stereospecifically assigned H_β resonances as well as details on the assignment process.This material is available free of charge via the Internet at http://pubs.acs.org.

Table 1. Stereospecific Assignment of H_β^{proR} and H_β^{proS} [a]

	δH_β^{proR} [ppm]/$^3J(H_\alpha,H_\beta^{proR})$ [Hz]	δH_β^{proS} [ppm]/$^3J(H_\alpha,H_\beta^{proS})$ [Hz]
E (5aa)	1.61 ± 0.02/8.2 ± 0.23	1.73 ± 0.03/5.2 ± 0.42
K (6aa)	1.38 ± 0.01/8.0 ± 0.37	1.45 ± 0.01/5.7 ± 0.47
L (5aa)	1.23 ± 0.03/8.4 ± 0.59	1.12 ± 0.06/5.9 ± 0.50
P (2aa)	1.58 ± 0.02/5.4 ± 0.03	1.94 ± 0.02/7.6 ± 0.40
Q (4aa)	1.55 ± 0.10/8.0 ± 0.45	1.65 ± 0.10/5.4 ± 0.21
R (2aa)	1.42 ± 0.01/7.8 ± 0.35	1.50 ± 0.01/5.5 ± 0.38

[a] Average chemical shifts and $^3J(H_\alpha H_\beta)$-coupling constants corresponding for H_β^{proR} and H_β^{proS} protons over the indicated number of residues in ubiquitin, pH 2, 8 M urea (the full data set can be found in the Supporting Information). $^3J(H_\alpha H_\beta)$-couplings were compared to the results of an inverse Pachler-type analysis of χ_1-populations to identify the matching ^{1}H signals.

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