Journal of Biomolecular NMR, **17:** 331–335, 2000.
KLUWER/ESCOM

Bridging the gap: A set of selective ^{1}H-^{15}N-correlations to link sequential neighbors of prolines

Mario Schubert, Linda J. Ball, Hartmut Oschkinat & Peter Schmieder*
Forschungsinstitut für Molekulare Pharmakologie, Alfred-Kowalke-Strasse 4, D-10315 Berlin, Germany

Received 1 May 2000; Accepted 5 June 2000

Key words: amino acid type selective experiments, proline, sequential assignment, triple-resonance

Abstract

Triple-resonance experiments are standard in the assignment of protein spectra. Conventional assignment strategies use ^{1}H-^{15}N-correlations as a starting point and therefore have problems when proline appears in the amino acid sequence, which lacks a signal in these correlations. Here we present a set of amino acid selective pulse sequences which provide the information to link the amino acid on either side of proline residues and thus complete the sequential assignment. The experiments yield amino acid type selective ^{1}H-^{15}N-correlations which contain signals from the amino protons of the residues either preceding or following proline in the amino acid sequence. These protons are correlated with their own nitrogen or with that of the proline. The new experiments are recorded as two-dimensional experiments and their performance is demonstrated by application to a 115-residue protein domain.

Introduction

Complete resonance assignment is a prerequisite for the study of structure and dynamics of biomolecules in solution (Wüthrich, 1986; Cavanagh et al., 1996). In [^{15}N,^{13}C]-labeled proteins a sequence specific assignment is nowadays almost exclusively achieved using 3D triple-resonance experiments (Kay et al., 1990; Montelione and Wagner, 1990; Clore and Gronenborn, 1991; Sattler et al., 1999). The starting point for the assignment usually exploits the ^{1}H-^{15}N correlation and thus the $^{1}H^{N}$ and ^{15}N chemical shifts. In three-dimensional experiments these chemical shifts are correlated with the $^{13}C^{\alpha}$, the $^{13}C^{\beta}$ or the $^{13}C'$ chemical shift of either the same or the preceding residue. By connecting the chemical shifts of all amino acid spin systems from a proper combination of 3D spectra one can achieve a sequence specific assignment. The high sensitivity, intrinsic resolution and ease of interpretation of triple resonance spectra have helped to establish this procedure as a standard strategy for the assignment of proteins, both in manual and automated resonance assignment (Moseley and Montelione, 1999).

The strategy fails, however, when a proline residue is reached in the amino acid sequence, since proline does not have an amino proton and therefore does not show correlations in any of the aforementioned spectra. A sequential assignment then has to rely on the observation of NOEs from proline to neighboring residues. This is not desirable, as the introduction of ambiguity from through-space interactions can lead to misassignments which are particularly detrimental to automated assignment procedures.

For the identification of residues preceding prolines, (i−1) residues, a proline selective HACA(CO)-(N) triple resonance experiment was proposed (Olejniczak and Fesik, 1994). The selection is based on the absence of an amide proton in proline and the experiment relies exclusively on scalar coupling. The (i−1) residues are characterized by their $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$ chemical shifts. Recently, a proline selective 3D HDHA(NCO)CAHA (Bottomley et al., 1999) was proposed, which uses the same selection mechanism. The chemical shifts of the proline $^{13}C^{\alpha}$ and $^{13}C^{\delta}$ are correlated with the $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$ chemical shift of

*To whom correspondence should be addressed. E-mail: schmieder@fmp-berlin.de

the preceding residue via scalar coupling. Kay and coworkers (Kanelis et al., 2000) proposed a combination of two non-selective 3D experiments for the assignment of proline-rich sequences. The $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$ chemical shifts are correlated with the nitrogen chemical shift of either the (i+1)-residue or both the i and (i+1)-residue. A potential drawback of these experiments is that they rely on the $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$ chemical shifts. The dispersion of the $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$ chemical shifts is bad compared with that of the $^{1}H^{N}$ and ^{15}N chemical shifts. In addition, the signals appear in the same region of the spectrum as the water signal and the experiments therefore require an excellent water suppression, in particular if they are to be used in automated assignment procedures.

Materials and methods

The spectra were recorded at 300 K on a Bruker DRX600 in standard configuration using an inverse triple resonance probe equipped with three-axes self shielded gradient coils. A 1.35 mM sample of the EVH1 domain from VASP uniformaly labeled with ^{15}N and ^{13}C in a 5 mm ultra-precision sample tube was used. Spectral widths were 3012 Hz (^{15}N) $\times$ 10000 Hz (^{1}H) in all cases. The Pro($N_{(i-1)}$, $H^{N}_{(i-1)}$ and the Pro($N_{(i+1)}$, $H^{N}_{(i+1)}$)-HSQC were acquired in 6 h using 128 scans per complex point (64 $\times$ 512 complex points). The Pro($N_{(i)}$, $H^{N}_{(i-1)}$)- and the Pro($N_{(i)}$, $H^{N}_{(i-1)}$)-HSQC were acquired in 6 h using 256 scans per complex point (32 $\times$ 512 complex points). The data were processed using a squared sinebell shifted by 90° as a window function in both dimensions and zero-filled to final sizes of 512 (t_1) $\times$ 1024 (t_2) real points, using XWINNMR from Bruker.

Results and discussion

We present here a set of experiments that yield ^{1}H-^{15}N correlations selective for residues that are sequential neighbors of proline residues. Two new pulse sequences are proposed that yield four two-dimensional spectra depending on which nitrogen shift is allowed to evolve. The new sequences are related to the amino acid type selective ^{1}H-^{15}N correlations that have recently been proposed (Schubert et al., 1999). The selection in this case, however, is not based on the MUSIC sequence (Schmieder et al., 1998)

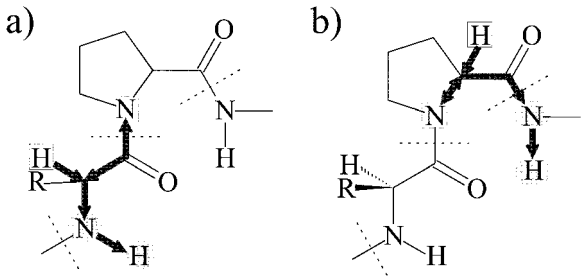

Figure 1. Schematic representation of the magnetization pathway selected by the new experiments. The flow of magnetization is indicated by the thick lines. (a) In the Pro($N_{(i-1)}$, $H^{N}_{(i-1)}$)- and Pro($N_{(i)}$, $H^{N}_{(i-1)}$)-HSQC the $^{1}H^{\alpha}$ of the (i−1) residue is excited and the magnetization is transferred via the $^{13}C^{\alpha}$ and $^{13}C'$ to the nitrogen of the proline and back to the $^{13}C'$ of the (i−1) residue. The magnetization is then transferred to the $^{15}N_{(i-1)}$ and the $^{1}H^{N}_{(i-1)}$ amide proton, which is detected. In the Pro($N_{(i+1),}H^{N}_{(i+1)}$)- and Pro($N_{(i),}H^{N}_{(i+1)}$)-HSQC magnetization originates from the $^{1}H^{\alpha}$ of the proline, is transferred via the $^{13}C^{\alpha}_{i}$ to the nitrogen of the proline and back to $^{13}C^{\alpha}_{i}$. From there it is transferred via the $^{13}C'$ to the $^{15}N_{(i+1)}$ and $^{1}H^{N}_{(i+1)}$, which is detected.

and the topology of the side chain, but on the absence of the amino proton in the proline residue. The Pro($N_{(i-1),}H^{N}_{(i-1)}$)-HSQC experiment yields exclusively ^{1}H-^{15}N signals of the residues preceding the prolines while the Pro($N_{(i)}$, $H^{N}_{(i-1)}$)-HSQC results in a correlation between the $^{1}H^{N}$ of the (i−1) residue and the proline nitrogen itself. The Pro($N_{(i+1)}$, $H^{N}_{(i+1)}$)-HSQC and the Pro($N_{(i),}H^{N}_{(i+1)}$)-HSQC result in the same type of correlations, only this time the $^{1}H^{N}$ of the residues succeeding proline, the (i+1) residue is involved. Taken together, these four experiments will not only identify the residues neighboring prolines but will also allow the (i−1) and (i+1) residues to be linked within the sequence.

The transfer of magnetization selected by the pulse sequences for the Pro($N_{(i-1)}$, $H^{N}_{(i-1)}$)-HSQC and the Pro(N_{i}, $H^{N}_{(i-1)}$)-HSQC is shown in Figure 1a. It can be described as

$$^{1}H^{\alpha}_{(i-1)} \rightarrow {}^{13}C^{\alpha}_{(i-1)} \rightarrow {}^{13}C'_{(i-1)} \rightarrow {}^{15}N_{(i)} \text{ (selection)}$$
$$\rightarrow {}^{13}C'_{(i-1)} \rightarrow {}^{13}C^{\alpha}_{(i-1)} \rightarrow {}^{15}N_{(i-1)} \rightarrow {}^{1}H^{N}_{(i-1)}$$

The pulse sequence is depicted in Figure 2a. The magnetization originates from the $^{1}H^{\alpha}$ of the (i−1) residue and is then transferred via the $^{13}C^{\alpha}$ and the $^{13}C'$ to the ^{15}N of proline (point a). During a delay τ_3 tuned to $1/2J_{HN}$ antiphase magnetization is created for all residues where an amide proton is present (point b). This magnetization is destroyed by the subsequent

composite pulse decoupling on the proton channel. Only proline magnetization, where no antiphase magnetization is created, is left. The magnetization is then transferred back to the $^{13}C'$ and the $^{13}C^{\alpha}$. Up to this point, the pulse sequence is identical with that of Olejniczak and Fesik (1994). But instead of returning to the $^{1}H^{\alpha}$ in an out-and-back manner, the magnetization is transferred to the nitrogen (point d) and the amide proton in the new sequence. To accomplish the transfer from $^{13}C^{\alpha}_{(i-1)}$ to $^{15}N_{(i-1)}$ a delay of 28 ms ($1/J_{CC}$) is used (Δ_3 in Figure 2a). If the (i−1) residue turns out to be a glycine, the resulting peaks will have opposite sign compared to all other types of amino acid, since the coupling between $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ is active during the delay for non-glycine amino acids. Two nitrogen nuclei are involved in the flow of magnetization. Depending on the nucleus that is allowed to evolve chemical shift, the resulting spectrum will show a correlation to the prolines own ^{15}N or to the ^{15}N of the (i−1) residue. To optimize the spectral window and the excitation of the nitrogen spins, the position of the nitrogen carrier is changed from 135 ppm to 120 ppm during the experiment (point c) and switched back at the end of the sequence.

The flow of magnetization that is selected by the pulse sequences for the Pro($N_{(i+1)}$, $H^{N}_{(i+1)}$)-HSQC and the Pro($N_{(i)}$, $H^{N}_{(i+1)}$)-HSQC is shown in Figure 1b. It can be described as

$$^{1}H^{\alpha}_{(i)} \rightarrow {}^{13}C^{\alpha}_{(i)} \rightarrow {}^{15}N_{(i)} \text{ (selection)} \rightarrow$$
$$^{13}C^{\alpha}_{(i)} \rightarrow {}^{13}C'_{(i)} \rightarrow {}^{15}N_{(i+1)} \rightarrow {}^{1}H^{N}_{(i+1)}$$

The pulse sequence is depicted in Figure 2b. The magnetization originates from the $^{1}H^{\alpha}$ of proline and is then transferred via the proline $^{13}C^{\alpha}$ to the proline ^{15}N (point a). During the delay $2\Delta_4$ the carbon magnetization is in the transverse plane and a selective REBURP pulse (Geen and Freeman, 1991) is applied to the $^{13}C^{\alpha}$ region of the prolines to refocus the coupling between $^{13}C^{\alpha}$ and the $^{13}C^{\beta}$. The length of the delay Δ_4 can then be optimized independent of the $^{1}J_{C^{\alpha}C^{\beta}}$ coupling. The selection of the proline ^{15}N is accomplished in the same way as with the sequence described above (point b). Then the magnetization is transferred back to the$^{13}C^{\alpha}$ and from there via the $^{13}C'$ and the nitrogen of the following residue (point d) to the amino proton. As with the other sequences, two nitrogen nuclei are involved in the magnetization transfer pathway and consequently two types of spectra can be recorded, depending on which nitrogen chemical shift is allowed to evolve. The same nitrogen carrier frequency jumps as in the other experiments are employed.

The sequences could also be executed as 3D experiments with two indirect dimensions recording nitrogen chemical shifts. Since the number of prolines in proteins is usually small, however, 2D experiments are generally sufficient to aid the assignment. Note that the amino protons of the residues next to proline are required in the new experiments. They are therefore not applicable to poly-proline sequences.

It should be noted that there is a potential drawback of the selection mechanism. The spectra are recorded in H_2O to allow for the suppression of amino acids other than proline. There is, however, an amount of 5–10% of D_2O in the sample used for the internal lock. Consequently, 5–10% of the amino acids have a deuteron bound to the nitrogen instead of a proton, irrespective of amino acid type. These residues can lead to low intensity break-through peaks (Figure 3b).

An application of the new pulse sequences to the EVH1 domain of VASP (Gertler et al., 1996; Ball et al., 2000) is shown in Figure 3. The protein has a length of 115 amino acids, 5 of which are prolines (residues 25, 30, 42, 56 and 77). The sequential neighbors of proline are identified from the Pro($N_{(i-1),}H^{N}_{(i-1)}$)-HSQC (Figure 3a) and Pro($N_{(i+1)}$, $H^{N}_{(i+1)}$)-HSQC (Figure 3b). The cross peaks of spectra (a) and (b) can be connected via the ^{15}N chemical shift of the proline common to both sets of experiments (a,b and c,d). This provides a method of linking the (i−1) and (i+1) residues on either side of the proline. In the Pro($N_{(i-1)}$, $H^{N}_{(i-1)}$)-HSQC only one peak with opposite sign appears. This identifies the residue immediately as Gly 29, since this is the only glycine in (i−1) position to a proline. The amino acid type represented by the other peaks can be identified using other amino acid selective ^{1}H-^{15}N correlations (Schubert et al., 1999). If an amino acid type appears only once as a neighbor of proline, this is then alone sufficient to assign the peak to a particular amino acid. In cases where there may be two or more assignment options, the combination of Pro(i−1) and Pro(i+1) correlations would provide a triplet of residues in the sequence, which would be more likely to be unique. An alternative approach is an assignment using conventional, 3D triple-resonance experiments. Here the residues neighboring proline will help to achieve a sequence specific assignment.

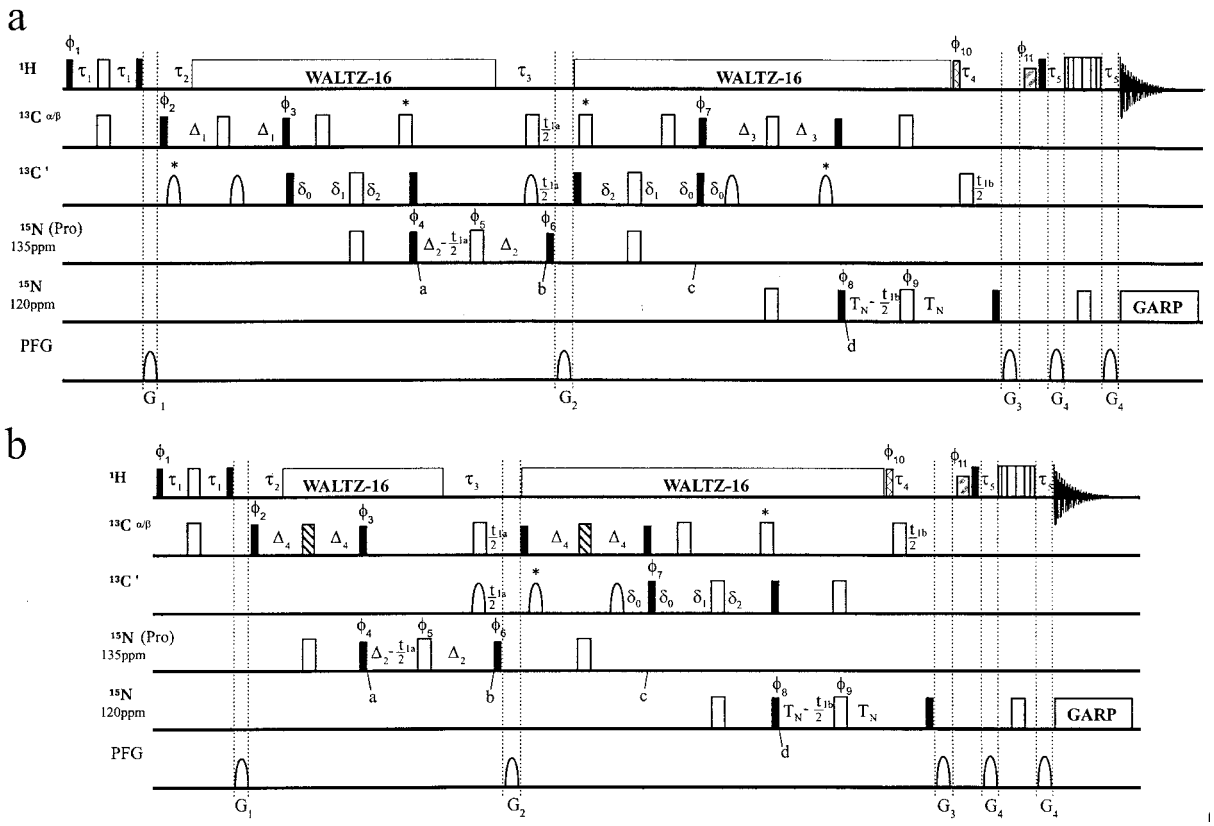

Figure 2. Pulse sequences of the new proline neighbor selective ^{1}H-^{15}N-correlations. The pulse sequences yield signals for residues preceding prolines (a) and residues following prolines (b). 90° and 180° pulses are represented by thin black filled and thick unfilled bars, respectively. The water-selective 90° ^{1}H flip-back pulse is represented by a gray and white striped thick bar, a hatched thin bar stands for a 90° flip-back ^{1}H pulse at the end of the ^{1}H-decoupling sequence. Magnetic field gradients as well as shaped 180° ^{13}C′ pulses are represented by sine shapes. Pulses applied at the ^{13}C$^{\alpha}$ or ^{13}C′ resonance frequencies were adjusted to provide a null at the corresponding ^{13}C′ or ^{13}C$^{\alpha}$ frequencies. The square ^{13}C$^{\alpha}$ 90° and 180° pulses were set to 54 μs and 48 μs, respectively. The square ^{13}C′ 90° and 180° pulses were set to 54 μs and 108 μs, respectively. The shaped 180° ^{13}C′ pulses were applied as G3 Gaussian cascades (Emsley and Bodenhausen, 1990) with a duration of 256 μs. Pulses with an asterisk are used to compensate off-resonance effects. The black and white striped thick bars stand for band-selective 180° REBURP pulses (Geen and Freeman, 1991) at 56 ppm with a duration of 1024 μs. Proton hard pulses were applied with 25 kHz field strength; WALTZ-16 decoupling (Shaka et al., 1983) of ^{1}H spins was achieved using a field strength of 3.1 kHz. The same field strength was used for the subsequent 90° flip-back ^{1}H pulse. The water-selective 90° square pulse had a duration of 1 ms. GARP-1 decoupling (Shaka et al., 1985) of ^{15}N was achieved using a field strength of 830 Hz. Water suppression was obtained using WATERGATE implemented with a 3-9-19 pulse (Sklenar et al., 1993). The gradients were applied as a sinusoidal function from 0 to π. The carrier frequencies were centered at ^{1}H = 4.8 ppm, ^{15}N = 120 and 135 ppm (see text), ^{13}C$^{\alpha}$ = 55 ppm and ^{13}C′ = 175 ppm. The following delays were used: τ_1 = 1.5 ms, τ_2 = 2.1 ms, τ_3 = 5.5 ms, τ_4 = 5.5 ms, τ_5 = 2.25 ms, Δ_1 = 3.5 ms, Δ_2 = 5.5 ms, Δ_3 = 14.2 ms, Δ_4 = 7 ms, δ_0 = 4.5 ms, δ_1 = 6.9 ms, δ_2 = 11.4 ms, T_N = 11 ms. Gradients had the following duration and strength: G_1 = 1 ms (y: 20 G/cm; z: 28 G/cm), G_2 = 1 ms (x: 25 G/cm; z: 35 G/cm), G_3 = (z: 31.5 G/cm), G_4 = 900 μs (z: 21 G/cm). To achieve quadrature detection in the indirect dimension the States-TPPI-States protocol (Marion et al., 1989) was used in all experiments. All spectra were processed using XWINNMR (Bruker AG). (a) **Pro($N_{(i-1)}$, $H^{N}_{(i-1)}$)-HSQC** (t_{1b} is incremented) and **Pro($N_{(i)}$, $H^{N}_{(i-1)}$)**-experiment (t_{1a} is incremented). The phase cycling was: ϕ_1 = y; ϕ_2 = 2 (y), 2 (−y); ϕ_3 = y; ϕ_4 = x; ϕ_5 = 32 (x), 32 (y), 32 (−x), 32 (−y); ϕ_6 = x, −x; ϕ_7 = 16 (x), 16 (−x); ϕ_8 = 4 (x), 4 (−x); ϕ_9 = 8 (x), 8 (y), 8 (−x), 8 (−y); ϕ_{10} = −y; ϕ_{11} = −x; ϕ_{rec} = (x, 2 (−x), x), 2 (−x, 2 x, −x), (x, 2 (−x), x), (−x, 2 x, −x), 2 (x, 2 (−x), x), 2 (−x, 2 x, −x), 2 (x, 2 (−x), x), (−x, 2 x, −x), (x, 2 (−x), x), 2 (−x, 2 x, −x), (x, 2 (−x), x). States-TPPI phase cycling was applied to ϕ_4 and ϕ_8 in the Pro($N_{(i-1)}$, $H^{N}_{(i-1)}$)-HSQC and the Pro($N_{(i)}$, $H^{N}_{(i-1)}$)-experiment, respectively. (b) **Pro($N_{(i+1)}$, $H^{N}_{(i+1)}$)-HSQC** (t_{1b} is incremented) and **Pro($N_{(i)}$, $H^{N}_{(i+1)}$)**-experiment (t_{1a} is incremented). The phase cycling was: ϕ_1 = y; ϕ_2 = 2 (y), 2 (−y); ϕ_3 = y; ϕ_4 = x; ϕ_5 = 32 (x), 32 (y), 32 (−x), 32 (−y); ϕ_6 = x, −x; ϕ_7 = 16 (x), 16 (−x); ϕ_8 = 4 (x), 4 (−x); ϕ_9 = 8 (x), 8 (y), 8 (−x), 8 (−y); ϕ_{10} = −y; ϕ_{11} = −x; ϕ_{rec} = (x, 2 (−x), x), 2 (−x, 2 x, −x), (x, 2 (−x), x), (−x, 2 x, −x), 2 (x, 2 (−x), x), 2 (−x, 2 x, −x), 2 (x, 2 (−x), x), (−x, 2 x, −x), (x, 2 (−x), x), 2 (−x, 2 x, −x), (x, 2 (−x), x). States-TPPI phase cycling was applied to ϕ_4 and ϕ_8 in the Pro($N_{(i+1)}$, $H^{N}_{(i+1)}$)-HSQC and the Pro($N_{(i)}$, $H^{N}_{(i+1)}$)-experiment, respectively.

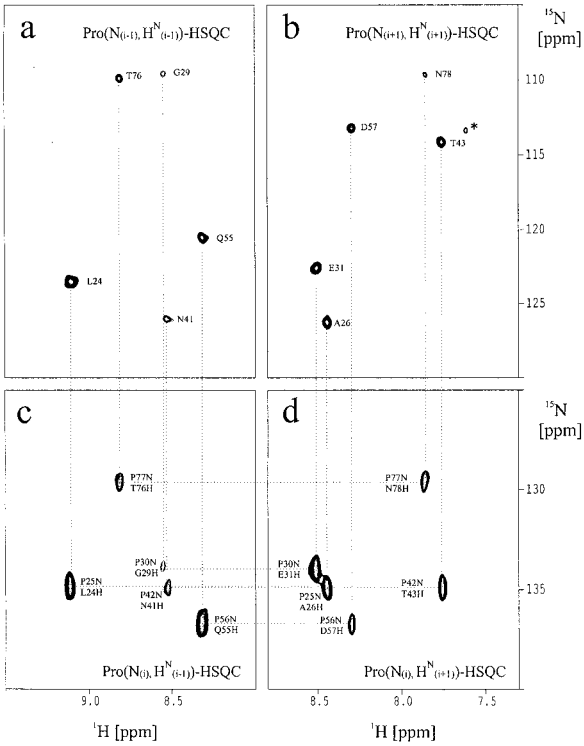

Figure 3. Spectra from the proline-selective experiments of the EVH1 domain from VASP. The EVH1 domain contains five prolines. (a) Pro($N_{(i-1)}$, $H^N_{(i-1)}$)-HSQC. All expected signals are visible. The signal of the glycine preceding Pro 30 is negative (see text). (b) Pro($N_{(i+1)}$, $H^N_{(i+1)}$)-HSQC. All five expected signals of residues following prolines are present. A peak from the C-terminal Gly 115, which shows a very intense peak in the ^{1}H-^{15}N-HSQC, is visible as a breakthrough peak. (c) Pro($N_{(i)}$, $H^N_{(i-1)}$)-HSQC. The spectrum shows correlations between the chemical shifts of $H^N_{(i-1)}$ and proline ^{15}N chemical shifts. All five $^{15}N_i/^1H_{(i-1)}$ correlations are present and the ^{15}N chemical shift of the prolines can be assigned to the corresponding peak in the P-(i−1)-HSQC. (d) The Pro($N_{(i)}$, $H^N_{(i+1)}$)-HSQC. All five $^{15}N_i/^1H_{(i+1)}$ correlations are visible and the ^{15}N chemical shift of the prolines can be assigned to the corresponding peak in the Pro($N_{(i+1)}$, $H^N_{(i+1)}$)-HSQC. Via the proline ^{15}N chemical shifts in spectra (c) and (d) a connection between residues directly preceding and following Pro can be established.

Conclusions

We have presented a new set of triple resonance experiments that enable the identification of sequential neighbors of proline. The (i−1) and (i+1) residues thus identified can be linked to pairs. This information will be helpful during resonance assignment of proteins, both as a starting point to identify sequential triplets of amino acids in the sequence and to bridge the gap left in sequential assignments performed using ^{1}H-^{15}N correlations.

Acknowledgements

Support from the Forschungsinstitut für Molekulare Pharmakologie is gratefully acknowledged. M.S. was supported by the DFG Graduiertenkolleg GRK 80 ‘Modellstudien’. The authors thank Thomas Jarchau for the preparation of the EVH1 sample. The work was supported by a grant of the BMBF (01 GG 9812, Leitprojekt ‘Proteinstrukturfabrik’).

References

Ball, L.J., Kühne, R., Schmieder, P., Hoffmann, B., Schneider-Mergener, J., Häfner, A., Hof, M., Wahl, M., Walter, U., Oschkinat, H. and Jarchau, T. (2000) *EMBO J.*, in press.

Bottomley, M.J., Macias, M.J., Liu, Z. and Sattler, M. (1999) *J. Biomol. NMR*, **13**, 381–385.

Cavanagh, J., Fairbrother, W.J., Palmer III, A.G. and Skelton N.J. (1996) *Protein NMR Spectroscopy*, Academic Press, San Diego, CA.

Clore, G.M. and Gronenborn, A.M. (1991) *Prog. NMR Spectrosc.*, **23**, 43–92.

Emsley, L. and Bodenhausen, G. (1990) *Chem. Phys. Lett.*, **165**, 469–476.

Geen, H. and Freeman, R. (1991) *J. Magn. Reson.*, **93**, 93–141.

Gertler, F.B., Niebuhr, K., Reinhard, M., Wehland, J. and Soriano, P. (1996) *Cell*, **87**, 227–239.

Kanelis, V., Donaldson, L., Muhandiram, D.R., Rotin, D., Forman-Kay, J.D. and Kay, L.E. (2000) *J. Biomol. NMR*, **16**, 253–259.

Kay, L.E., Ikura, M., Tschudin, R. and Bax, A. (1990) *J. Magn. Reson.*, **89**, 496–514.

Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.

Montelione, G.T. and Wagner, G. (1990) *J. Magn. Reson.*, **87**, 183–188.

Moseley, H.N. and Montelione, G.T. (1999) *Curr. Opin. Struct. Biol.*, **9**, 635–642 and references cited therein.

Olejniczak, E.T. and Fesik, S.W. (1994) *J. Am. Chem. Soc.*, **116**, 2215–2216.

Sattler, M., Schleucher, J. and Griesinger, C. (1999) *Prog. NMR Spectrosc.*, **34**, 93–158.

Schmieder, P., Leidert, M., Kelly, M.J.S. and Oschkinat, H. (1998) *J. Magn. Reson.*, **131**, 199–201.

Schubert, M., Smalla, M., Schmieder, P. and Oschkinat, H. (1999) *J. Magn. Reson.*, **141**, 34–43.

Shaka, A.J., Keeler, J., Frenkiel, T. and Freeman, R. (1983) *J. Magn. Reson.*, **52**, 335–338.

Shaka, A.J., Barker, P.B. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.

Sklenar, V., Piotto, M., Leppik, R. and Saudek, V. (1993) *J. Magn. Reson.*, **A102**, 241–245.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York, NY.