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Journal of Magnetic Resonance 182 (2006) 325–329
JMR Journal of Magnetic Resonance
www.elsevier.com/locate/jmr

Communication

^{13}C direct detected COCO-TOCSY: A tool for sequence specific assignment and structure determination in *protonless* NMR experiments

Stéphane Balayssac, Beatriz Jiménez, Mario Piccioli *

Magnetic Resonance Center (CERM), Department of Chemistry, University of Florence, Via Luigi Sacconi 6, 50019 Sesto Fiorentino (FI), Italy

Received 6 February 2006; revised 7 June 2006
Available online 17 July 2006

Abstract

A novel experiment is proposed to provide inter-residue sequential correlations among carbonyl spins in ^{13}C detected, *protonless* NMR experiments. The COCO-TOCSY experiment connects, in proteins, two carbonyls separated from each other by three, four or even five bonds. The quantitative analysis provides structural information on backbone dihedral angles ϕ as well as on the side chain dihedral angles of Asx and Glx residues. This is the first dihedral angle constraint that can be obtained via a *protonless* approach. About 75% of backbone carbonyls in Calbindin D_{9K}, a 75 aminoacid dicalcium protein, could be sequentially connected via a COCO-TOCSY spectrum. 49 $^3J_{C'C'}$ values were measured and related to backbone ϕ angles. Structural information can be extended to the side chain orientation of aminoacids containing carbonyl groups. Additionally, long range homonuclear coupling constants, $^4J_{CC}$ and $^5J_{CC}$, could be measured. This constitutes an unprecedented case for proteins of medium and small size.

Keywords: ^{13}C direct detection; NMR spectroscopy; COCO-TOCSY; Dihedral angle restraints; ^{13}C homonuclear coupling constants; Pulse sequences

1. Introduction

Direct detection of ^{13}C spins is an established protocol to study proteins in solution, when the observation of ^1H NMR resonances is hampered [1–4]. This methodology, termed *protonless* NMR approach, has been used, either alone or in combination with conventional ^1H based experiments, for a number of macromolecules such as systems in chemical exchange [5], partially or completely unfolded proteins [4], paramagnetic [6] and metal substituted proteins [7]. Due to the quadratic dependence of signal linewidths from the gyromagnetic ratio, γ, of the relaxing spin, heteronuclear detection compensates for additional contributions to relaxation [8]. Within this frame, a number of pulse sequences have been developed to perform extensive NMR assignment exclusively based on C–C or C–N coherence transfers and signal assignments [4].

The observation of inter-residue connectivities using *protonless* NMR experiment is, however, a critical aspect of the approach. The available experiments suffer from low sensitivity due to the weak ^1J and ^2J $^{13}C^{\alpha}$–$^{15}N^{H}$ coupling constants, so far exploited to obtain sequential connectivities in the CANCO experiment [4,9]. Experiments based on long range coupling constants between atoms of two neighbouring aminoacids could be an alternative route for sequential assignment. Here we propose the use of the COCO-TOCSY experiment, based on the homonuclear Hartman–Hahn cross polarization (TOCSY), which gives inter-residue connectivities between adjoin carbonyl spins, via a $^3J_{C'(i)-C'(i+1)}$ magnetization transfer. The interest of this experiment is twofold: (i) to provide a coherence transfer pathway for inter-residue connectivities different from CANCO, (ii) to use ^{13}C detected experiments to obtain structural information on dihedral angle restraints. This

* Corresponding author. Fax: +39 055 457 4271.
E-mail address: piccioli@cerm.unifi.it (M. Piccioli).

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doi:10.1016/j.jmr.2006.06.021

is particularly relevant because, until now, only sparse structural information can be obtained via ^{13}C direct detection.

Furthermore, $^{3}J_{C'C'}$ can be related to ϕ backbone angle. Proton detected experiments have been already developed to measure such coupling [10]. The ^{13}C detected quantitative measurement of small $^{3}J_{C'C'}$ coupling constants would therefore constitute the first structural constraint that, besides chemical shifts, can be obtained from ^{13}C detected NMR experiments. This would significantly extend the application of the *protonless* approach, up to now mainly used for assignment purposes.

Additionally, also long range $^{3}J_{C'C\gamma}$ and even $^{4}J_{C'C\delta}$ and $^{5}J_{C'(i-1)C\delta(i)}$ will be observed and related to side chain conformation of Asx (i.e. Asp or Asn) and Glx (i.e. Glu or Gln) aminoacids [11].

2. Results and discussion

The COCO-TOCSY sequence is shown in Fig. 1. Excitation and chemical shift evolution of carbonyls are followed by a spin lock scheme [12]. During the long isotropic mixing period (120 ms) each carbonyl correlates with the previous (C'_{i-1}) and the successive one (C'_{i+1}). An IPAP building block [13,14] is added before the acquisition to remove the strong C^{α}–C' couplings in the acquisition dimension [4].

Experiments were recorded on a 1.5 mM sample of $^{13}C/^{15}N$-enriched dicalcium Calbindin D_{9k} (Ca_2Cb), a 75 aminoacid protein containing two EF-hand domains [15], for which the complete NMR assignment and the solution structure are already known [16]. The α-helix scaffold originates small $^{3}J_{C'C'}$ values [17] and small spreading of C' signals. About 75% of backbone carbonyl sequential connectivities were identified in Calbindin D_{9k} from the COCO-TOCSY. For 34 aminoacids, the spectrum showed resolved cross peaks with both the previous and the following residues, thus giving a straightforward pattern, as pictured in Fig. 2. Most of the missing signals cannot be observed due to very small differences between the carbonyl chemical shifts. The full list of connectivities obtained is reported in Supplementary Material.

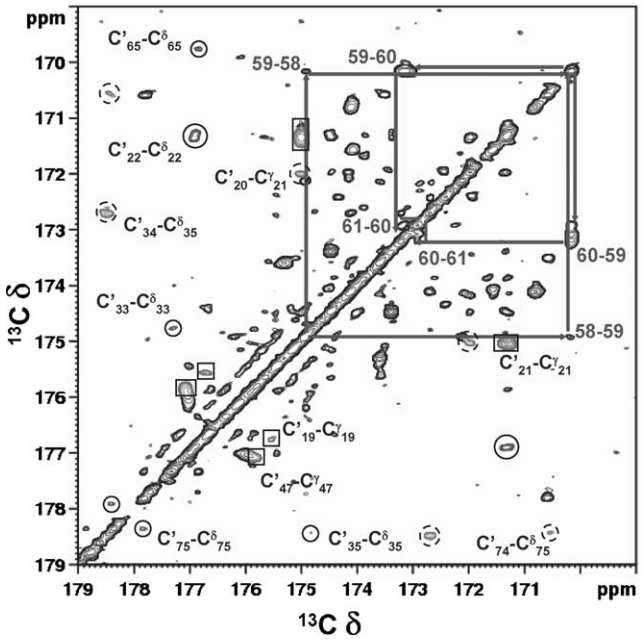

Fig. 2. Portion of the COCO-TOCSY spectrum of Ca_2Cb (1.5 mM) recorded at 300 K on a Bruker Avance 500 (11.7 T) equipped with a triple resonance, 5-mm, ^{1}H cryoprobe with a cooled ^{13}C preamplifier (TCI) and z-axis gradients. Cross peaks arising from couplings involving side chain carbons are marked with squares for $^{3}J_{C'C\gamma}$, circles for $^{4}J_{C'C\delta}$, and dashed circles for inter residue $^{4}J_{C'C\gamma}$ and $^{5}J_{C'C\delta}$. A 400×512 data point matrix was acquired using 256 scans each fid. Total experimental time was 43 h. Recycle delay was 1 s. A 3.1 kHz spin-lock was applied for 120 ms. The full spectrum is shown in Supplementary Material.

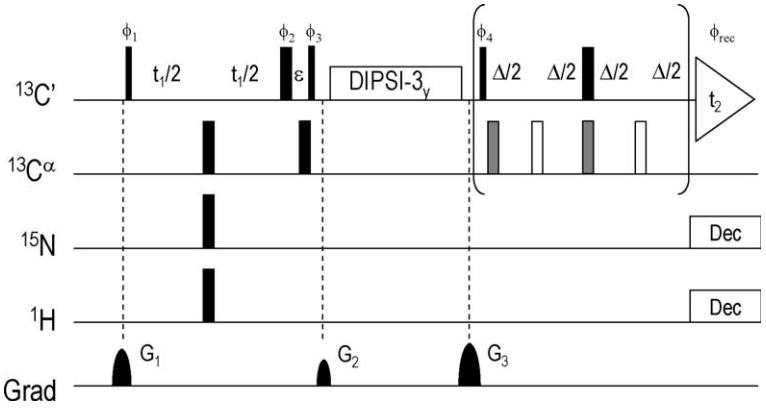

Fig. 1. COCO-TOCSY pulse sequence. Narrow and wide bars represent 90° and 180° pulses. Q5 (333 μs) and Q3 (220 μs) Gaussian cascades [23] were used for ^{13}C pulses. ε is a small delay which corresponds to the initial t_1 value. Phases were x unless indicated. DIPSI-3$_y$ was applied. Two experiments were collected to acquire separately the in phase and the anti phase component of the strong C^{α}–C' homonuclear coupling. Pulses indicated with open bars were used to obtain the in-phase component, while shaded pulses were used for the anti-phase ($\Delta = 4.55$ ms). Phase cycles were $\phi_1 = x, -x$; $\phi_2 = 4x$, $4(-x)$; $\phi_3 = 2x, 2(-x)$; $\phi_4 = 4x, 4(-x)$ or $\phi_4 = 4(-y), 4y$ to obtain the in-phase or the anti-phase component, respectively, $\phi_{rec} = x, -x, -x, x, -x, x, x, -x$. Phase sensitive was obtained by incrementing ϕ_1 in a States-TPPI manner [24]. G_1, G_2 and G_3 were 0.6, 1 and 3 ms, respectively, with field strength of 21, 11.2, and 42 G/cm. ^{1}H and ^{15}N were decoupled during acquisition using waltz16 [25] and garp4 [26], respectively.

The COCO-TOCSY also connects backbone carbonyls with C' spins from side chains. Four out of six C′–C$^{\gamma}$ intra-residue correlations of Asx (i.e. Asp or Asn) residues were assigned (Fig. 2, squares) and provided the corresponding $^{3}J_{C'C\gamma}$ coupling constant (Asp 19, Asn 21, Asp 47 and Asp 58). Coupling constants of higher order could also be observed: 8 out of 17 C′–C$^{\delta}$ correlations of Glx residues were identified (Fig. 2, circles) and the corresponding $^{4}J_{C'C\delta}$ coupling constants were estimated (see Supplementary Material). Connectivities between side chain carbonyls and the backbone carbonyl of the previous residue have been also found (Fig. 2, dashed circles), i.e. $^{4}J_{CC}$ Pro 20 C′–Asn 21 C$^{\gamma}$, or Leu 46 C′–Asp 47 C$^{\gamma}$, and even $^{5}J_{CC}$, such as Thr 34 C′–Glu 35 C$^{\delta}$ and Ser 74 C′–Gln 75 C$^{\delta}$. To our knowledge, this is the first time that such couplings have been observed in proteins. Indeed, the spin-lock of carbonyl spins provides a dramatic simplification of coupling patterns and makes possible the identification of remote couplings. The chemical shift difference between backbone and side chain carbonyls is such that these cross peaks resonate in a well-resolved spectral region, thus facilitating their assignment.

The quest for structural information fully based on ^{13}C direct detection is important in order to move from *protonless* NMR assignment to *protonless* solution structure determination [18]. Because no heteronuclear coherence transfer is involved in this experiment, observed cross peak intensities only depend on the extent of the scalar coupling between the two carbonyl spins and on their relaxation properties [10]. Different C′ spins will have different relaxation rates, which in principle can be completely accounted for when cross peaks are scaled by the corresponding diagonal peak [10,19]. In COCO-TOCSY, the resolution of the 2D experiment is such that diagonal peaks can be quantitatively measured only for few resonances. A third dimension could be encoded to resolve overlaps, however this would introduce at least one additional coherence transfer step that would modulate peak intensities. We used the few well resolved diagonal peaks to obtain the average intensity of a diagonal peak and the estimate of the deviation from the mean value, which was found to be ±25%. These values were used to obtain a quantitative measurement of $^{3}J_{C'C'}$, for which a ±50% uncertainty was given. In Fig. 3, backbone $^{3}J_{C'C'}$ values are shown with respect to the Karplus curve previously parametrized by Hu and Bax [17]. Only two peaks deviate from the fitting, they arise from connectivites between Lys 41–Gly 42 and Ser 74–Gln 75. The former residues are part of the linker region between the two EF-hand domains, which has been reported to undergo conformational equilibria [20] and to be prone to structural rearrangements [21] while the latter constitute the C-terminal sequential connectivity. These peaks fit in the Karplus curve when cross peak intensities are scaled down according to the relaxation rates of individual C′ spins [18]. The latter have been measured independently (L. Poggi, personal communication). Also the two $^{5}J_{CC}$ observed for Ca_2Cb are due to residues placed in the flexible linker region of the protein (Glu 35) or in the C-term region. It is therefore likely that the slower relaxation rates of these spins contributed to the identification of such weak couplings. This validates the idea that a single COCO-TOCSY experiment is sufficient to obtain, via a *protonless* approach, constraints on the dihedral angle ϕ. The very small values of observed couplings and the lower sensitivity of ^{13}C direct detection with respect to conventional ^{1}H direct detection are such that the conversion of observed couplings into structural restraints must be performed with a relatively high tolerance. Better results are likely to be obtained in proteins presenting a β-scaffold folding, in which couplings are expected to be larger than in the present case.

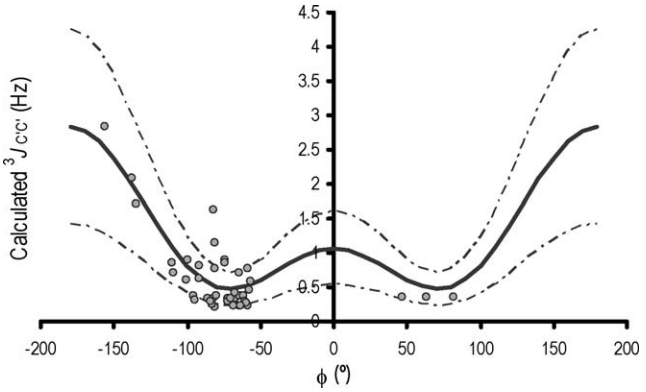

Fig. 3. Relationship between $^{3}J_{C'C'}$ values and ϕ angles (measured from 1KSM.pdb [16]) and a best fit Karplus curve according to the parametrization previously reported ($^{3}J_{C'C'} = 1.33\ \cos^2\ \phi - 0.88\cos\phi + 0.62$) [17]. Upper and lower limit curves are calculated with a ±50% uncertainty of experimental data, arising from the propagation of the experimental uncertainty of cross peak and of diagonal peak intensities.

Available structural information is not only limited to backbone C′–C′ but can be extended to the side chain orientation of aminoacids containing carbonyl groups, and an estimate of torsion angles χ_1 can be given. About 4.5 Hz are expected for $^{3}J_{C'C\gamma}$ of Asp or Asn in *trans* conformation, (χ_1 ca. −60°) while values of 0.7 Hz are estimated for a *gauche* motif [17]. Consistently, side chains of Asn 21 and Asp 47, which are in *trans*, provide the largest cross peaks in COCO-TOCSY, while Asp 19 and Asp 58, which have intermediate conformations ($\chi_1 = -148.4°$, −23.7°, respectively), show much weaker peak intensities. The C′–C$^{\gamma}$ cross peaks of the two metal binding groups, Asp 54 and Asn 56, are not observed in the spectrum, in agreement with their *gauche* conformation [16].

There are very few examples of $^{4}J_{CC}$ in the literature [11], and no case is reported for proteins. For saturated chains, it has been observed in small molecules, that $^{4}J_{CC}$ are related to the two torsion angles defined by the five carbon atoms of the chain and large $^{4}J_{CC}$ are observed when both torsion angles are close to 180°. This is the case, in Ca_2Cb, of Glu 11, Gln 22, Glu 27, Gln 33, Glu 35, Glu 48, Glu 65 and Gln 75, for which both torsion angles are in the range 180° ± 20 ° and for which $^{4}J_{C'C\delta}$ are observed.

3. Conclusions

The efficiency of C′–C′ transfer via isotropic mixing is not a novel concept and pulse sequences have been developed [10,22] which successfully exploit this coherence transfer pathway to perform a sequential assignment [17]. However, when the COCO-TOCSY block is encoded in a ^{13}C-observed experiment, the direct acquisition of ^{13}C′ provides a method to perform sequential assignment which has improved sensitivity with respect to the established coherence transfer pathways relying on the CANCO experiment [9]. The relevant features of COCO-TOCSY are twofold: on one hand, connecting each C′ spin with both adjoin spins, i.e. (C'_{i-1}) and (C'_{i+1}), substantially increases the opportunities for a complete assignment. A combination of CBCACO and COCO-TOCSY is, in principle, sufficient for a complete sequential assignment. On the other hand, a semiquantitative analysis of cross peak intensities provides dihedral angle restraints on the backbone ϕ angles, as well as on the side chain χ_1 of Asx and Glx and χ_2 of Glx residues. As a consequence, information on secondary structure are available also in a *protonless* approach. This finding would be extremely useful when addressing early stages of folding processes or when dealing with systems characterized by dramatic exchange broadening of H^N signals. Given the small coupling constants, observed cross peaks are rather weak and therefore the quantitative analysis is hindered by the low S/N ratio affecting most of the cross peaks. A relatively high tolerance would be needed to use ϕ angle restraints obtained from COCO-TOCSY within structure calculations. However this is the first case in which such information is obtained using *protonless* NMR experiments.

Acknowledgments

S.B. is a Marie Curie Host Fellow Early Stage Research Training (EST) (Contract MEST-CT-2004-504391). B.J. is a Marie Curie Intra European Fellow (EIF) (Contract MEIF-CT-2005-515039). The project is funded by European Union, ''Structural Proteomics IN Europe, SPINE'' (Contract QLG2-CT-2002-00988).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmr.2006.06.021.

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