In-Cell NMR Spectroscopy

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^{13}C Direct-Detection Biomolecular NMR Spectroscopy in Living Cells**

Ivano Bertini, Isabella C. Felli, Leonardo Gonnelli, Vasantha Kumar M. V., and Roberta Pierattelli*

In-cell NMR experiments of labeled biomolecules have been shown to be quite informative about the structure and dynamics of proteins in an environment similar to the natural one.[1–7] The main approaches have been 1) to overexpress proteins in prokaryotic cells,[6,8] 2) to inject labeled proteins in oocytes,[9–11] and 3) to introduce into eukaryotic cells labeled proteins through a target sequence.[12] The most widely used experimental strategy consists in exploiting ^{15}N isotopic labeling to collect ^{1}H–^{15}N correlation experiments to monitor changes in chemical shift and to map biochemical processes and interactions.[13–15] ^{1}H–^{13}C correlation experiments are less popular because of the large background signals complicating the analysis of the spectra. Indeed, even not considering the background signals resulting from the labeling of other cellular components besides the protein of interest, the 1% natural abundance of ^{13}C is sufficient to provide strong signals. This can be partly overcome by correlating ^{13}C with other heteronuclei, as in the case of ^{1}H-detected triple resonance experiments that have been used to obtain the sequential assignment of proteins in-cell.[7,16] In other cases, selective ^{13}C enrichment has been used, with particular success when focusing on histidines[17] and methyl groups.[18]

We have recently pursued the exploitation of ^{13}C direct detection in solution for biomolecular NMR applications.[19] Thanks to the increase in instrumental sensitivity as well as to improved experimental schemes, ^{13}C-detected 2D experiments can be acquired in a very short time (a few minutes).[20] Herein, we report the advantages and limits in terms of applications of ^{13}C-detected experiments acquired on prokaryotic cells containing overexpressed ^{13}C,^{15}N-enriched proteins. In particular, we show that such experiments are quite informative for unfolded parts of proteins for which ^{1}H–^{15}N NMR experiments are less informative.

The studied protein is yeast Cox17 (69 amino acids), which is largely unfolded when the seven cysteine residues present in its primary sequence are reduced and largely folded when four of them form interhelix disulfide bonds with a coil-helix-coil-helix fold.[21] Comparative experiments are reported for folded yeast Atx1 (72 amino acids)[22] and for human α-synuclein (140 amino acids), a natively disordered protein which maintains this state also in-cell.[23]

The ^{1}H–^{15}N correlation NMR spectrum[24] of Cox17 in *Escherichia coli* cells provides a fingerprint of the protein in-cell (Figure 1 A). At first sight the spectrum appears to be that of an unfolded protein as the spreading of the ^{1}H signals is small. This spectrum is close to that of the purified protein in a reducing environment (20 mM dithiothreitol), in which the cysteine residues of the protein are reduced (Figure 1 B).[21] However, the poor chemical shift dispersion as well as the increased linewidths typical of in-cell experiments only allow us to resolve a limited number of ^{1}H–^{15}N correlations. The ^{13}C′–^{15}N correlation (CON) spectrum[20,25] in-cell shows a number of signals (Figure 1 C) that is smaller than that of the reduced species in solution (Figure 1 D). The ^{13}C$^{\alpha/\beta}$–^{13}C′ correlation (CACO[20,26] and CBCACO[27]) spectra, which allow us to obtain through simple 2D experiments information on other backbone nuclei (C$^{\alpha}$s) and on side chains (C$^{\beta}$s),

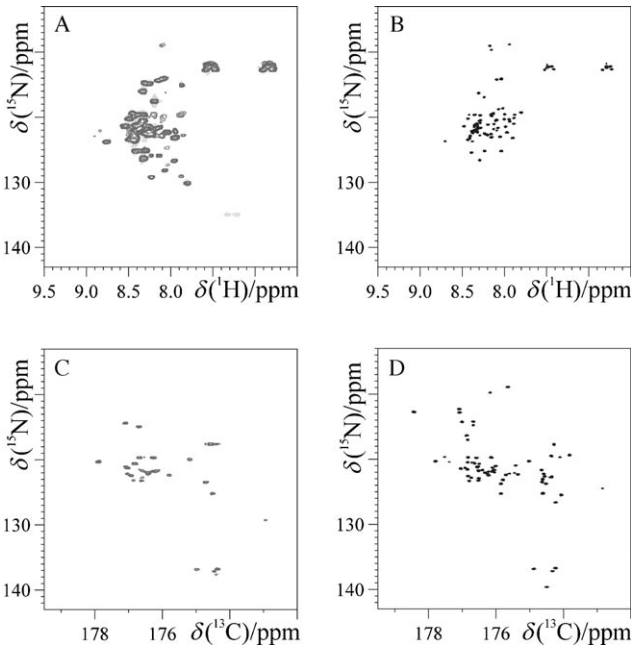

Figure 1. A, B) ^{1}H–^{15}N and C,D) ^{13}C′–^{15}N correlation spectra acquired on A, C) *E. coli* cells overexpressing yeast Cox17 and B, D) on the purified protein. The details of sample conditions and experimental parameters are reported in the Supporting Information.

[*] Prof. I. Bertini, Prof. I. C. Felli, Prof. R. Pierattelli
CERM and Department of Chemistry "Ugo Schiff"
University of Florence
Via Luigi Sacconi 6, 50019 Sesto Fiorentino, Florence (Italy)
Fax: (+39) 055-457-4271
E-mail: ivanobertini@cerm.unifi.it
Dr. L. Gonnelli, V. Kumar M. V.
CERM, University of Florence
Via Luigi Sacconi 6, 50019 Sesto Fiorentino, Florence (Italy)

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also show the same behavior (Figure S1 in the Supporting Information).

We then assigned the reduced purified protein in solution through a combined use of ^{1}H- and ^{13}C-detected multidimensional NMR experiments (97% H^N, 98% N, 97% C′, 100% C^α, 100% C^β nuclei, as reported in the Supporting Information and in the BMRB 17205). By simple comparison of the spectra acquired in-cell with those acquired in solution, the detected signals in-cell can be assigned to the residues in the first part and in the final part of the primary sequence (1–36, 65–69). Interestingly, in the fully reduced state of the protein in solution these residues are characterized by negative heteronuclear ^{1}H–^{15}N NOEs (Figure S2 in the Supporting Information). The latter are very sensitive indicators of fast local motions and clearly show that this part of the molecule is characterized by fast motions (^{1}H–^{15}N NOEs < 0, effective local correlation time $< 10^{-9}$). The central part of the protein, which cannot be detected through ^{13}C-detected experiments in-cell, instead has a much lower extent of fast internal motions (^{1}H–^{15}N NOEs > 0 and < 0.5), which indicates a partially folded conformation still not characterized by a stable 3D structure.

To establish the performance of in-cell NMR experiments on proteins characterized by different motional properties,[28] the same ^{13}C-detected NMR experiments were also collected on a protein of similar size to Cox17 but characterized by a well-defined 3D structure, Atx1,[22] as well as on a well-known intrinsically disordered protein, human α-synuclein,[29,30] characterized by similar motional properties to the initial and final parts of fully reduced Cox17. The results obtained clearly show that Atx1 largely maintains its 3D fold in cells, as evidenced by the very similar ^{1}H–^{15}N NMR spectrum with respect to the purified protein (Figure 2 A,B). On the other hand, the ^{13}C-detected CON experiment, recorded in parallel to the ^{1}H–^{15}N NMR experiment, only shows a few correlations, most of which can be ascribed to background signals (Figure 2 E). However, all the expected ^{13}C–^{15}N correlations (Figure 2 D) could be detected by recording the CON experiment on the corresponding cell extracts (Figure 2 F), that is, on a sample with the same protein concentration as in Figure 2 E but in a less viscous and inhomogeneous solution.[31]

Therefore, the lack of signals in the experiments acquired in cells does provide an indication of a significant increase in the effective correlation time of Atx1 in-cell.[32] Comparison of the same spectra acquired on proteins with larger molecular mass (Figure S3 in the Supporting Information) in solution shows that an apparent molecular mass more than twice that of Atx1 justifies a loss of signals in the ^{13}C-detected experiments.[33] Therefore, the comparison provides indirect evidence of the effect of the in-cell environment on the motional properties of Atx1.

On the contrary, the CON spectra acquired on *E. coli* cells overexpressing human α-synuclein (Figure 2 K) provide NMR correlations for the large majority of the residues of the protein, thanks to the faster motional properties of intrinsically disordered proteins with respect to globular ones.[30] The improved chemical shift dispersion of the ^{13}C–^{15}N correlations (Figure 2 K) with respect to ^{1}H–^{15}N ones (Figure 2 H), however, permits resolution of a larger number of signals and thus provides additional atomic resolution information.

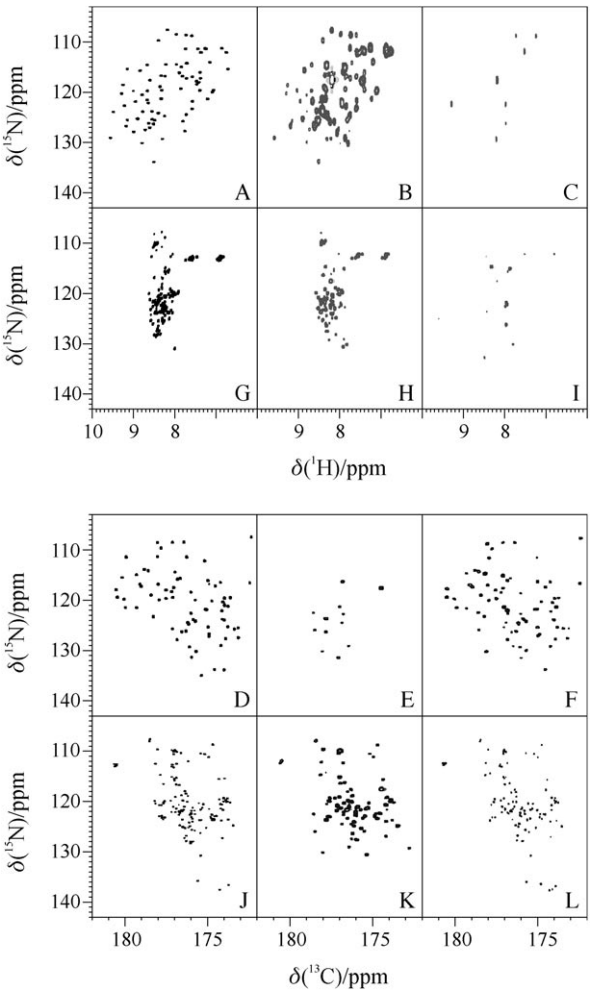

Figure 2. ^{1}H–^{15}N correlation (A–C, G–I) and ^{13}C′-^{15}N correlation (D–F, J–L) spectra recorded on yeast Atx1 (A–F) and human α-synuclein (G–L) either on the purified proteins (A, D, G, J) or in-cell (B, E, H, K). Protein leakage was excluded for acquiring control ^{1}H–^{15}N correlation experiments for Atx1 (C) and α-synuclein (I). The ^{13}C′–^{15}N correlation experiments collected for the cell extracts are also shown for Atx1 (F) and α-synuclein (L). The sample conditions and experimental details are reported in the Supporting Information. (D) is taken from Ref. [31] and (G) and (J) from Ref. [29].

This comparison between the relative performance of the most simple ^{1}H- and ^{13}C-detected 2D correlation experiments with ^{15}N acquired in cells overexpressing either a well-folded globular protein (Atx1) or an intrinsically disordered protein (α-synuclein) clearly shows that their combined use provides valuable complementary information to characterize the structural and dynamic properties of proteins in cells. The contribution of ^{13}C–^{15}N correlation experimental results is particularly useful for the characterization of unfolded components in the cells, as it allows one to highlight them from folded components and to extend the number of residues for which atomic resolution information can be obtained. Furthermore, as demonstrated by the case of Cox17

in-cell, the combination of ^{1}H–^{15}N and ^{13}C–^{15}N 2D NMR experiments enables the identification, within this largely disordered protein, of two distinct modules characterized by different local flexibility.

In conclusion, the exclusively heteronuclear NMR experiments proposed here for in-cell studies provide clean spectra of very mobile parts of proteins, and contain information on all backbone heteronuclei as well as on side chains in the simple 2D mode, without the need to employ specifically labeled amino acids in the culture medium to simplify the spectra.[18] The relatively high ^{13}C natural abundance does not pose problems, as heteronuclear–heteronuclear transfer steps reduce contributions from background signals. The application of these experiments to prokaryotic and particularly to eukaryotic cells may enable one to follow the maturation process of proteins expressed as unfolded in the cytoplasm, or the occurrence of posttranslational modifications that often involve disordered protein modules.

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