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**NMR Characterization of Long-Range Contacts in Intrinsically  
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Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

NMR Characterization of Long-Range Contacts in Intrinsically Disordered Proteins from Paramagnetic Relaxation Enhancement in  $^{13}\text{C}$  Direct-Detection Experiments / Mateos, Borja; Konrat, Robert; Pierattelli, Roberta\*; Felli, Isabella C.. - In: CHEMBIOCHEM. - ISSN 1439-4227. - STAMPA. - 20:(2019), pp. 335-339. [10.1002/cbic.201800539]

*Availability:*

This version is available at: 2158/1145992 since: 2021-03-29T13:12:06Z

*Published version:*

DOI: 10.1002/cbic.201800539

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# ChemBioChem

## NMR characterization of long-range contacts in intrinsically disordered proteins from paramagnetic relaxation enhancement in <sup>13</sup>C direct-detected experiments --Manuscript Draft--

|   |   |
|---|---|
| <b>Manuscript Number:</b>                         | cbic.201800539R1  |
| <b>Article Type:</b>                              | Communication   |
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| <b>Keywords:</b>                                  | intrinsically disordered proteins, IDP, <sup>13</sup> C-direct detection, PRE   |
| <b>Abstract:</b>                                  | <p>Intrinsically disordered proteins (IDPs) carry out many biological functions. They lack a stable 3D structure and are able to adopt many different conformations in dynamic equilibrium. The interplay between local dynamics and global rearrangements is key for their function. A widely used NMR experimental approach to study long-range contacts in IDPs exploits paramagnetic effects and <sup>1</sup>H detected experiments are generally used to determine paramagnetic relaxation enhancement (PRE) for amide protons. However, under physiological conditions exchange broadening hampers the detection of solvent exposed amide protons reducing the content of information available. Here we present an experimental approach based on direct carbon detection of PRE that provides improved resolution, reduced sensitivity to exchange broadening and complementary information deriving from the use of different starting polarization sources.</p> |
| <b>Response to Reviewers:</b>                     | <p>Authors: Borja Mateos, Robert Konrat, Roberta Pierattelli and Isabella C. Felli<br/>Title: NMR characterization of long-range contacts in intrinsically disordered proteins (IDPs) from paramagnetic relaxation enhancement (PRE) in <sup>13</sup>C direct detected experiments</p> <p>To the Editorial Office,</p> <p>Thanks for the positive evaluation of the manuscript.<br/>Following the suggestion of the reviewer we have included a final comment to stress the challenges that are being addressed through the proposed experimental NMR approach (page 4, left column, initial part of the concluding paragraph). The general context was already described in the initial paragraphs (page 1).<br/>Enclosed please find also high resolution versions of all figures as well as the Supporting Information.</p> <p>Best regards,</p> <p>Roberta Pierattelli and Isabella C. Felli</p>  |
| <b>Section/Category:</b>                          |   |
| <b>Additional Information:</b>                    |   |
| <b>Question</b>                                   | <b>Response</b>   |
| Submitted solely to this journal?                 | Yes   |
| Has there been a previous version?                | Yes   |
| Please state previous 1) Manuscript ID            | Manuscript number: 201805724  |

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| <p>and 2) journal. 3) If the paper was reviewed, please include a point-by-point response to the reviewer comments. as follow-up to "Has there been a previous version?"</p> | <p>Angewandte Chemie</p> <p>The detailed response to the reviewers' comments has been uploaded.</p> |
| <p>Do you or any of your co-authors have a conflict of interest to declare?</p>  | <p>No. The authors declare no conflict of interest.</p>   |
| <p>Animal/tissue experiments?</p>  | <p>No</p>   |

## COMMUNICATION

# NMR characterization of long-range contacts in intrinsically disordered proteins from paramagnetic relaxation enhancement in $^{13}\text{C}$ direct-detected experiments

Borja Mateos<sup>[a]</sup>, Robert Konrat<sup>[a]</sup>, Roberta Pierattelli<sup>\*[b]</sup> and Isabella C. Felli<sup>\*[b]</sup>

**Intrinsically disordered proteins (IDPs) carry out many biological functions. They lack a stable 3D structure and are able to adopt many different conformations in dynamic equilibrium. The interplay between local dynamics and global rearrangements is key for their function. A widely used NMR experimental approach to study long-range contacts in IDPs exploits paramagnetic effects and  $^1\text{H}$  detected experiments are generally used to determine paramagnetic relaxation enhancement (PRE) for amide protons. However, under physiological conditions exchange broadening hampers the detection of solvent exposed amide protons reducing the content of information available. Here we present an experimental approach based on direct carbon detection of PRE that provides improved resolution, reduced sensitivity to exchange broadening and complementary information deriving from the use of different starting polarization sources.**

Intrinsically disordered proteins (IDPs) are highly flexible proteins that exist as ensembles of rapidly interconverting structures. The large conformational space they sample allows them to serve as interaction hubs and to recruit different binding partners and therefore to modulate many biological events<sup>[1–5]</sup>. These proteins are present in many cellular pathways and their deregulation is often associated with a propensity to develop diseases such as cancer and neurodegenerative disorders.

The lack of a stable 3D structure resulting from fast local motions of the polypeptide chain makes them exquisitely suited systems to be studied by solution-state NMR spectroscopy, the only technique able to access high resolution information on highly flexible molecules<sup>[6–8]</sup>. The structural information content available from proton-proton dipole-dipole interactions (i.e. NOE-derived distance information), widely used in folded proteins, is very poor in IDPs due to their highly dynamic nature<sup>[10]</sup>. Therefore paramagnetic effects deriving from the presence of an unpaired electron<sup>[9]</sup> located in a strategic position along the primary sequence in an IDP have been widely used to determine spatially encountering regions within 5–25 Å<sup>[10–15]</sup>. Due to the large gyromagnetic ratio of the electron, even low-populated

intramolecular contacts can be probed. The most widely used method to determine paramagnetic relaxation enhancements consists in the measurement of the attenuation of intensity of cross peaks in 2D  $\text{H}^{\text{N}}$  detected  $^1\text{H}$ - $^{15}\text{N}$  correlation experiments due to paramagnetic interaction. However, approaching physiological conditions amide protons of residues in largely exposed protein backbones experience efficient exchange with the solvent protons; this causes signal broadening which may prevent the detectability of the signals or affect the accuracy of intensity measurements. An approach to overcome this drawback is to focus on aliphatic  $^1\text{H}$ - $^{13}\text{C}$  correlation experiments<sup>[16–18]</sup>, such as methyl groups, but in IDPs these signals are often not well resolved, reducing the effectiveness of the method. Therefore, alternative methods to exploit the potential of PRE in IDPs are welcome.

Direct  $^{13}\text{C}$ -detected experiments are widely used to study IDPs due to the large dispersion of the C'-N correlations in 2D CON spectra<sup>[19–23]</sup> and to the negligible sensitivity to solvent-exchange processes<sup>[24–26]</sup>, quite pronounced in disordered regions. In addition  $^{13}\text{C}$ -detected schemes have been originally developed for the study of paramagnetic metalloproteins<sup>[27,28]</sup> to take advantage of the reduced sensitivity of heteronuclei to dipolar contributions to paramagnetic relaxation that depend on the gyromagnetic ratio of the nuclear spins one is looking at. Indeed, several cases have appeared in the literature in which exclusively heteronuclear (*protonless*) experiments were used to recover high resolution information in regions close to a paramagnetic center while protons escaped detection<sup>[19,28–30]</sup>. On the same grounds this property of paramagnetic dipolar contributions to nuclear relaxation was used to access electron-nucleus distance information in complementary ranges around the paramagnetic centre by determining both  $^1\text{H}$  PREs, through  $^1\text{H}$  detected experiments, and  $^{13}\text{C}$  PREs, through  $^{13}\text{C}$  detected *protonless* experiments<sup>[31]</sup>. This approach was then extended to the characterization of protein-protein interfaces in folded proteins<sup>[32]</sup> and an initial study showed its potential for IDPs by monitoring variations of NMR signal intensities occurring upon  $\text{Cu}^{2+}$  binding in  $\alpha$ -synuclein<sup>[33]</sup>.

Several features thus make heteronuclear approaches very promising for the study of compact states of IDPs near physiological conditions<sup>[26,34]</sup>. Here we would like to evaluate the potential of different variants of the CON experiment for the determination of PREs in IDPs. To this end we used the well-characterized and paradigmatic IDP osteopontin (OPN), a biomedically relevant protein involved in the pathology of diverse physiological processes as well as in tumour progression and metastasis in various types of cancer<sup>[35,36]</sup>.

The sequence specific assignment of the 220 amino acids long polypeptide at 293 K is available<sup>[37]</sup> (BMRB ID 15519) and  $^1\text{H}$  detected PREs, determined for 4 single cysteine mutants through the MTSL tag (*S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate), revealed a central part of the

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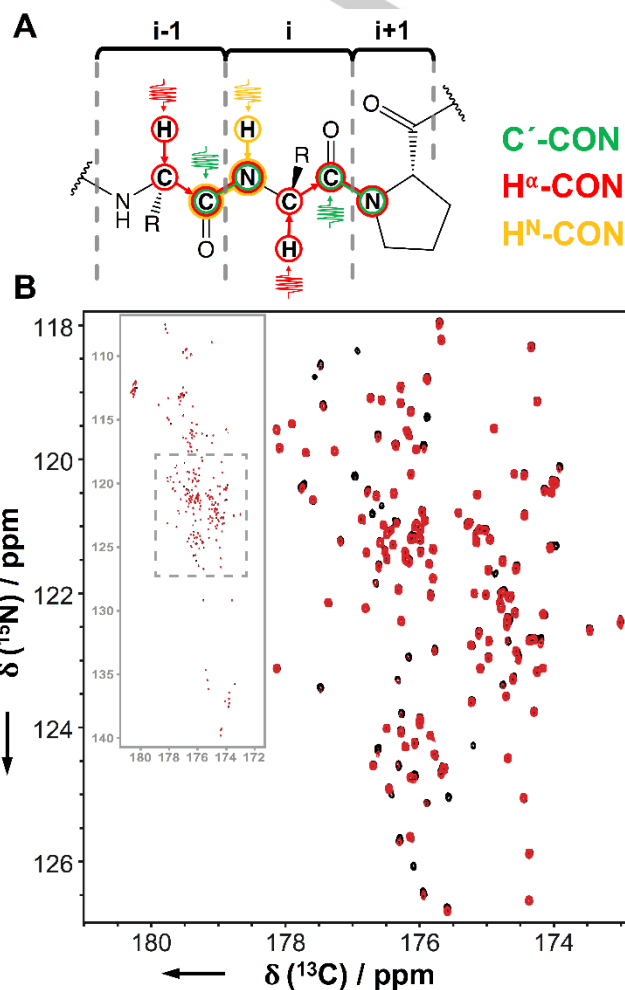
## COMMUNICATION

protein forming a so-called compact state<sup>[38–41]</sup>, which was difficult to detect through other observables<sup>[37]</sup>. Thus it represents a nice system to evaluate the contribution of the CON-based approach to the characterization of compact states in IDPs near physiological conditions.

By using <sup>13</sup>C detection schemes we extended the sequence specific assignment to higher temperatures (310 K). A series of 3D <sup>13</sup>C detected experiments<sup>[34,42,43]</sup> [(H)CBCACON, (H)CBCANCO, (H)CCCON and (H)COCON]) allowed us to obtain an essentially complete assignment (99.1%, BMRB ID 27443) of backbone heteronuclei and of C<sup>β</sup>, including 12 prolines (which constitute 5.5 % of the primary sequence). The secondary structure propensity (SSP) values using this extended data set are reported in Figure S1.

The CON spectrum was then used as template to determine PRE effects (Figure 1). The vast majority of the signals can be resolved allowing us to obtain a nearly complete sampling along the primary sequence. Several variants of the CON experiment differing in the starting polarization source (C', H<sup>α</sup>, H<sup>N</sup>, schematically shown in Figure 1A) were used to modulate the experimental sensitivity, the extent of the paramagnetic effects as well as the completeness of the data. The experiments in which only non-exchangeable nuclear spins are exploited in the magnetization transfer pathway (C'-CON and H<sup>α</sup>-CON) allow us to study IDPs also at high pH and temperature values. Moreover, C'- and H<sup>α</sup>-starting polarization sources allow us to detect signals involving proline peptide bonds and thus offer excellent data completeness. In addition, they also provide complementary PRE information. Indeed the C'-CON, one of the basic 2D *protonless* experiments, does not exploit <sup>1</sup>H in any of the steps of the coherence transfer pathway. Therefore the observed PREs depend on electron-nucleus dipole-dipole interactions sensed by carbonyl carbon atoms (C' PREs) which are smaller compared to those sensed by protons by a factor  $(\gamma_C/\gamma_H)^2$  at the same electron-nucleus distance<sup>[28,31,44]</sup>. This implies that residues close to the paramagnetic tag are affected to a lower extent by paramagnetic relaxation enhancement and show a better signal-to-noise ratio compared to the variants that exploit <sup>1</sup>H as a starting polarization source. In other words, the C'-CON experiment allows us to monitor effects at shorter distances from the paramagnetic tag, and potentially provides new restraints for ensemble calculations in IDPs. Proton-start experiments (H<sup>α</sup>-CON and H<sup>N</sup>-CON) instead are more sensitive to paramagnetic effects because protons, with their high gyromagnetic ratio compared to carbon, are reintroduced in the coherence transfer pathway to transfer coherence from protons to heteronuclei (<sup>13</sup>C or <sup>15</sup>N). In all cases, the inter-scan delays were set long enough (4–6 s) to avoid undesirable effects that may affect PRE determination<sup>[17,45]</sup>. The H<sup>α</sup>-CON still permits to monitor proline residues as in the C'-CON but it provides a measure of the PRE effects sensed by H<sup>α</sup> nuclear spins (H<sup>α</sup> PREs). The H<sup>N</sup>-CON experiment, instead, provides less complete information because it does not allow us to monitor prolines and it is more vulnerable to solvent exchange effects that may become prominent approaching physiological temperature and pH. However, the H<sup>N</sup>-CON is still useful for the determination of PRE effects in IDPs because of the different magnetization transfer pathway exploited in this experiment. Indeed, cross peaks observed in the different variants of the experiment (C'-CON, H<sup>α</sup>-CON and H<sup>N</sup>-CON) share common frequencies (C'-

N<sub>i+1</sub>); however, their intensities are modulated by different magnetization transfer pathways, as indicated in



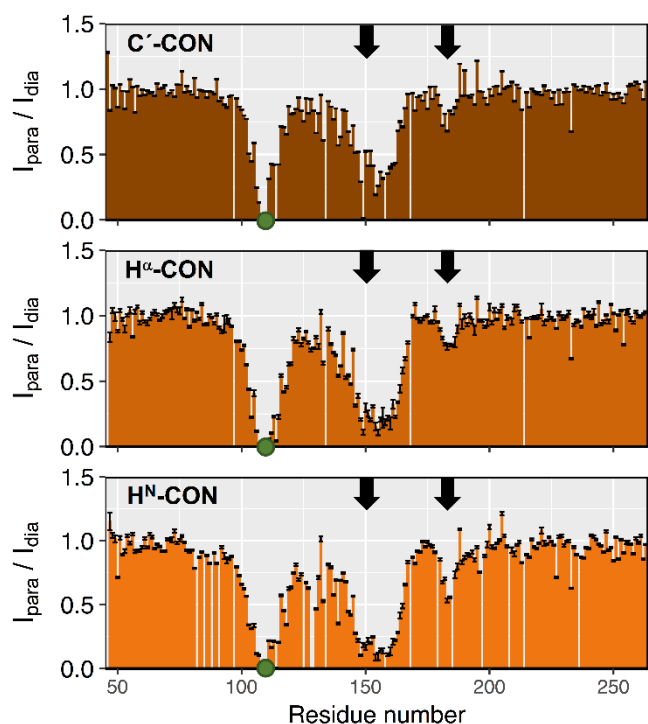
**Figure 1.** <sup>13</sup>C direct-detected PRE experiments on OPN. (A) Scheme indicating the nuclear spins involved in the polarization transfer pathway of the three experiments recorded: C'-CON (green), H<sup>α</sup>-CON (red) and H<sup>N</sup>-CON (orange); the symbols with the arrows indicate the starting polarization source. (B) C'-CON spectra of OPN (mutant S188C) acquired on the diamagnetic (black) and paramagnetic (red) states of the protein. The inset shows the complete spectrum indicating the region that has been expanded to highlight the high resolution of cross peaks that can be obtained in the 2D mode.

Figure 1A. Thus, each experiment reports about different sites: the H<sup>α</sup>-CON experiment reports about the H<sup>α</sup> of amino acid “i” and the H<sup>N</sup>-CON about the H<sup>N</sup> of amino acid “i+1”. The combined analysis of H<sup>N</sup>-CON and H<sup>α</sup>-CON spectra is thus useful to obtain more robust information and eventually identify false positive PRE effects arising from experimental errors.

As an example of the long-range information that can be collected through the CON-based approach, the set of data measured for the S108C OPN mutant is shown in Figure 2. PREs could be determined for the vast majority of the amino acids by acquiring the experiments on the paramagnetic and on the diamagnetic state of S108C OPN mutant and by measuring intensity ratios for all the signals. The profiles significantly deviate

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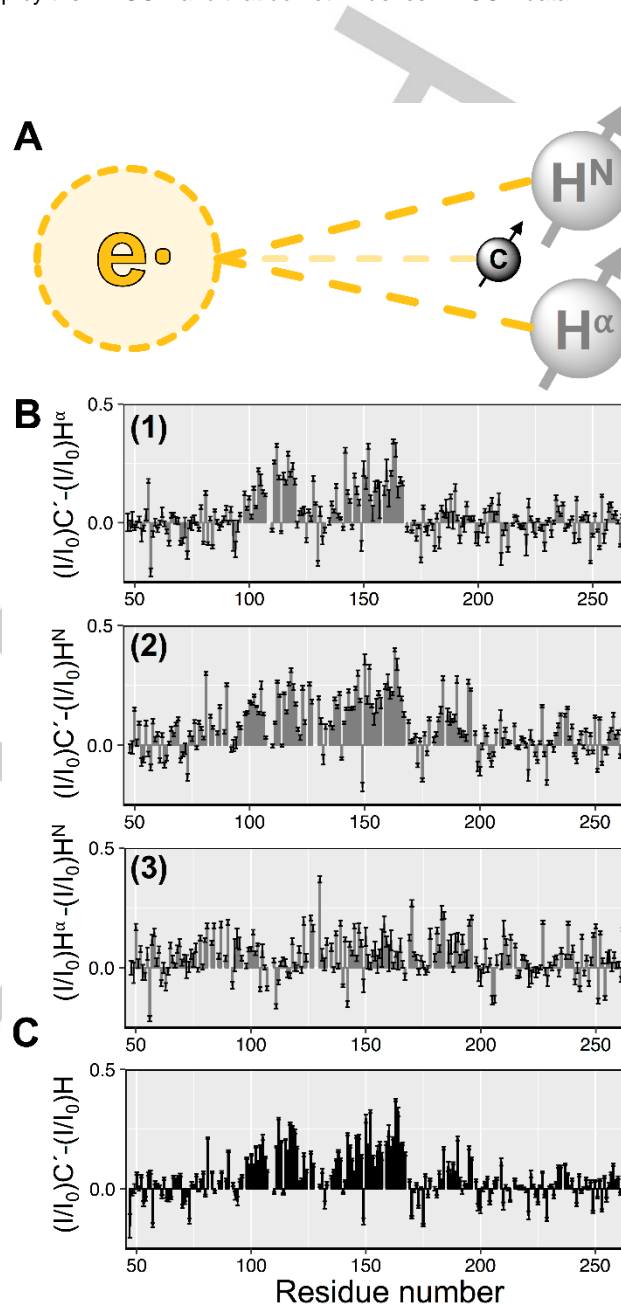
from what could be expected from a completely random coil polypeptide and allow us to identify long



**Figure 2.** PRE intensity profiles determined through the CON-based approach exploiting different starting magnetization sources: C'-CON (top panel), H $\alpha$ -CON (middle panel), HN-CON (bottom panel). The green circle indicates the position of the spin-label (S108C). Black arrows indicate the observed long-range contacts with the spin-label.

range contacts with two regions distant in the primary sequence from the paramagnetic tag (Figure 2). The reduced sensitivity of the  $^{13}\text{C}$  detected *protonless* variant (C'-CON) to paramagnetic relaxation enhancement can be readily inferred by the smaller effects observed for residues immediately preceding and following the paramagnetic tag in the primary sequence. In addition, comparing C'-CON and H $\alpha$ -CON derived long range PREs, it appears that they both highlight the same regions of the protein (indicated by arrows) with the former showing less pronounced effects, in agreement with  $^{13}\text{C}$  nuclear spins sensing similar paramagnetic effects at shorter distances from the paramagnetic center with respect to  $^1\text{H}$  nuclear spins. Still it is interesting to note that the long-range contact between tag at position 108 and the region encompassing residues 180-190 is observed through the C'-CON variant, confirming that conformations in which these two protein regions are in close contact are indeed significantly populated. Finally, it is interesting to compare the PREs observed through the H $\alpha$ -CON and HN-CON, which both exploit  $^1\text{H}$  as a starting polarization source. The higher amount of missing data in the HN-CON profile, largely due to the presence of proline residues, indicates how abundant prolines are in the vicinity of the compact state and thus the importance of accessing experimental information to characterize prolines as well. In addition, the HN-CON profiles are less uniform with respect to the H $\alpha$ -CON profiles, a feature that could be due to additional

solvent-mediated relaxation enhancement effects that are picked up by the H $^{\text{N}}$ -CON and that do not influence H $\alpha$ -CON data.



**Figure 3.** (A) Scheme representing the combined information of H $^{\text{N}}$  and H $\alpha$  (average) respect to C'. Note that proton is more sensitive to paramagnetic effects. (B) Comparison of intensity ratios determined through the CON-based approach exploiting different starting polarization sources (C', H $\alpha$  and H $^{\text{N}}$ ). The spin-label is located in position 108. Panel (1) represents the intensity ratio difference between C' and H $\alpha$ ; panel (2) between C' and H $^{\text{N}}$ ; and panel (3) between H $^{\text{N}}$  and H $\alpha$ . (C) Scheme representing the respective gyromagnetic ratios and its distance dependence to the unpaired electron of the spin label (MTSL).

The complementary information achieved through the three different experimental variants can also be inspected by reporting the differences in the observed ratios (Figure 3). It is interesting to note that the major differences between the  $^{13}\text{C}$  protonless variant ( $\text{C}'\text{-CON}$ ) and the two variants exploiting  $^1\text{H}$  ( $\text{H}^\alpha\text{-CON}$  and  $\text{H}^{\text{N}}\text{-CON}$ ) are observed for residues 100-170 which form the molten-globule core of the compact state in OPN. This central part of the protein seems to play a major role for the function of OPN, since it contains the RGD motif (responsible for binding to integrin) and has previously been shown to bind to heparin<sup>[38]</sup>. Confirmation of the above findings was obtained applying a similar strategy to three other single cysteine mutants of OPN harboring the paramagnetic tag at positions 54, 188 and 247 (Figure S2). While the mutants with tags at positions 54 and 247 do not show pronounced long-range contacts, the mutant with the tag at position 188 shows long-range contacts with the region 125-160, supporting the results obtained through the mutant harboring the paramagnetic tag at position 108.

Although there is ample NMR evidence for the existence of a compact state in OPN (comprising the segment 100-190), detailed structural information is still missing and a large number of spin-labels is in principle necessary to properly describe the conformational ensemble of the protein and the structural elements present (work in progress). For  $^1\text{H}$ -detected experiments it was proposed to set one spin-label every 15 residues<sup>[13]</sup> to contrast the broadening effect in the vicinity of MTSL. We anticipate that our approach might reduce the number of required spin-labels to obtain valuable distance constraints in future structure calculations. Finally, the PRE data obtained through the CON-based approach ( $\text{C}'$ ,  $\text{H}^\alpha$  and  $\text{H}^{\text{N}}$ ) can be used to calculate Pearson-correlation maps, which have been demonstrated to be a useful tool to qualitatively describe compact states in IDPs<sup>[15]</sup>. The combined use of the different CON-based experiments ( $\text{C}'$ ,  $\text{H}^\alpha$  and  $\text{H}^{\text{N}}$ ) will further improve the accuracy of the obtained correlation maps (Figure S3).

**Concluding, the proposed approach addresses several of the challenges involving the structural and dynamic characterization of IDPs: it enables the study of IDPs near physiological conditions providing complete coverage along the primary sequence with an excellent resolution and it provides complementary distance information exploiting paramagnetic relaxation enhancements involving nuclear spins with different gyromagnetic ratios ( $^{13}\text{C}$  and  $^1\text{H}$ ).** In case of OPN, the CON-based approach to measure PRE effects in IDPs allowed us to monitor essentially all amino acids and to access PREs not only for  $\text{H}^{\text{N}}$ , but also for  $\text{H}^\alpha$  and  $\text{C}'$ , providing a complete coverage along the whole polypeptide chain (about 60% coverage was achieved through the  $\text{H}^{\text{N}}$  detected 2D HSQC experiments<sup>[39]</sup>). This superior coverage allowed us to probe an increased number of long range contacts in IDPs under nearly physiological condition<sup>[42]</sup> offering also potential for in-cell applications<sup>[25,46]</sup>. Certain residue types like glycine, serine and threonine have a strong water-amide exchange broadening at physiological conditions which causes poor signal-to-noise in  $^1\text{H}$ -detected experiments and prevents the determination of PREs. However, these residues, in addition to prolines, are highly abundant in IDPs and their role in local dynamics and phosphorylation events is key for the function of many IDPs<sup>[47,48]</sup>. Therefore, the possibility to monitor PRE effects also for these

residues is highly valuable. This opens new opportunities for the study of IDPs at near-physiological conditions. Finally, the complementary information of the PRE sensed by diverse backbone nuclei will potentially facilitate the ensemble calculation in IDPs, expanding the available toolbox to study these challenging systems.

## Experimental Section

Samples of  $^{13}\text{C}$   $^{15}\text{N}$  labeled OPN as well as of 4 single cysteine mutants, with cysteines introduced at positions 54, 108, 188 and 247, were expressed and purified as previously reported<sup>[39]</sup>. For NMR experiments samples were in 50 mM sodium phosphate, 50 mM sodium chloride and pH 6.5, with a protein concentration of about 2 mM for OPN and of 0.8-1 mM for each of the single cysteine mutants. 1%  $\text{D}_2\text{O}$  was sufficient for the lock signal. The paramagnetic tag, S-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate (MTSL), was attached to each of the single cysteine mutants as previously described<sup>[39]</sup>. The reduced diamagnetic form was obtained by addition of 4-fold excess of ascorbic acid respect to the protein concentration.

The  $^{13}\text{C}$  detected NMR experiments for sequence specific assignment at 310 K were acquired on a 16.4 T Bruker AVANCE NEO NMR spectrometer equipped with a cryogenically cooled triple resonance probehead optimized for  $^{13}\text{C}$  direct detection. Typical experiments and parameters for  $^{13}\text{C}$  detected biomolecular NMR experiments were used as detailed in Table S1. For the determination of PRE values,  $\text{C}'\text{-CON}$ ,  $\text{H}^\alpha\text{-CON}$  and  $\text{H}^{\text{N}}\text{-CON}$  experiments were acquired. Detailed parameters are reported in Table S2.

## Acknowledgements

The support and the use of resources of the CERM/CIRMMP centre of Instruct-ERIC, a Landmark ESFRI project, is gratefully acknowledged. This work has been supported in part by iNEXT (EC Horizon 2020 contract # 653706) and by a grant of the Fondazione CR Firenze to RP. BM is grateful to the NGP-net COST action (ID: 38248) for funding a short term mission to the Magnetic Resonance Center of the University of Florence.

**Keywords:** intrinsically disordered proteins (IDPs) •  $^{13}\text{C}$ -direct detection • NMR spectroscopy • paramagnetic relaxation enhancement (PRE) • long-range contacts

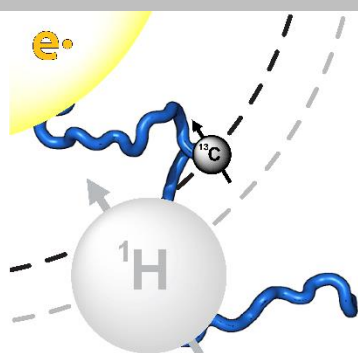
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## COMMUNICATION

PREs provide unique information about long range contacts in IDPs. Here we propose  $^{13}\text{C}$  detected NMR experiments to facilitate structural investigations approaching physiological conditions.



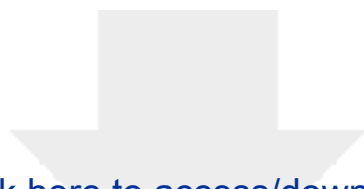
*Borja Mateos, Robert Konrat, Roberta Pierattelli\* and Isabella C. Felli\**

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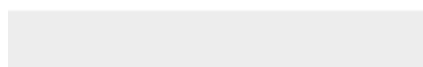
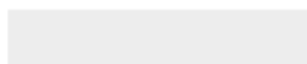
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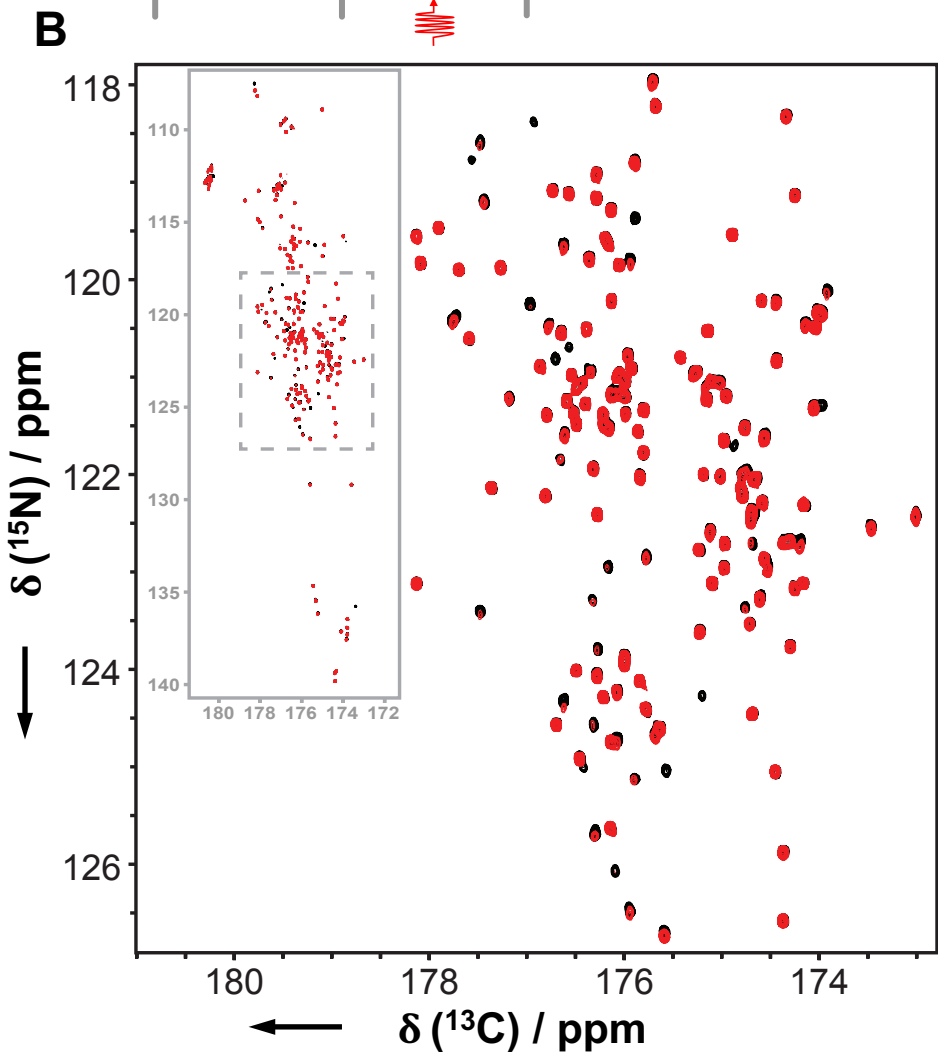
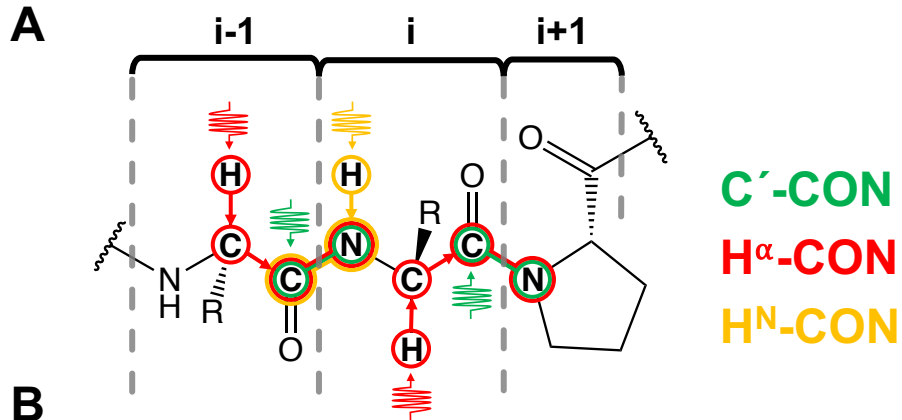
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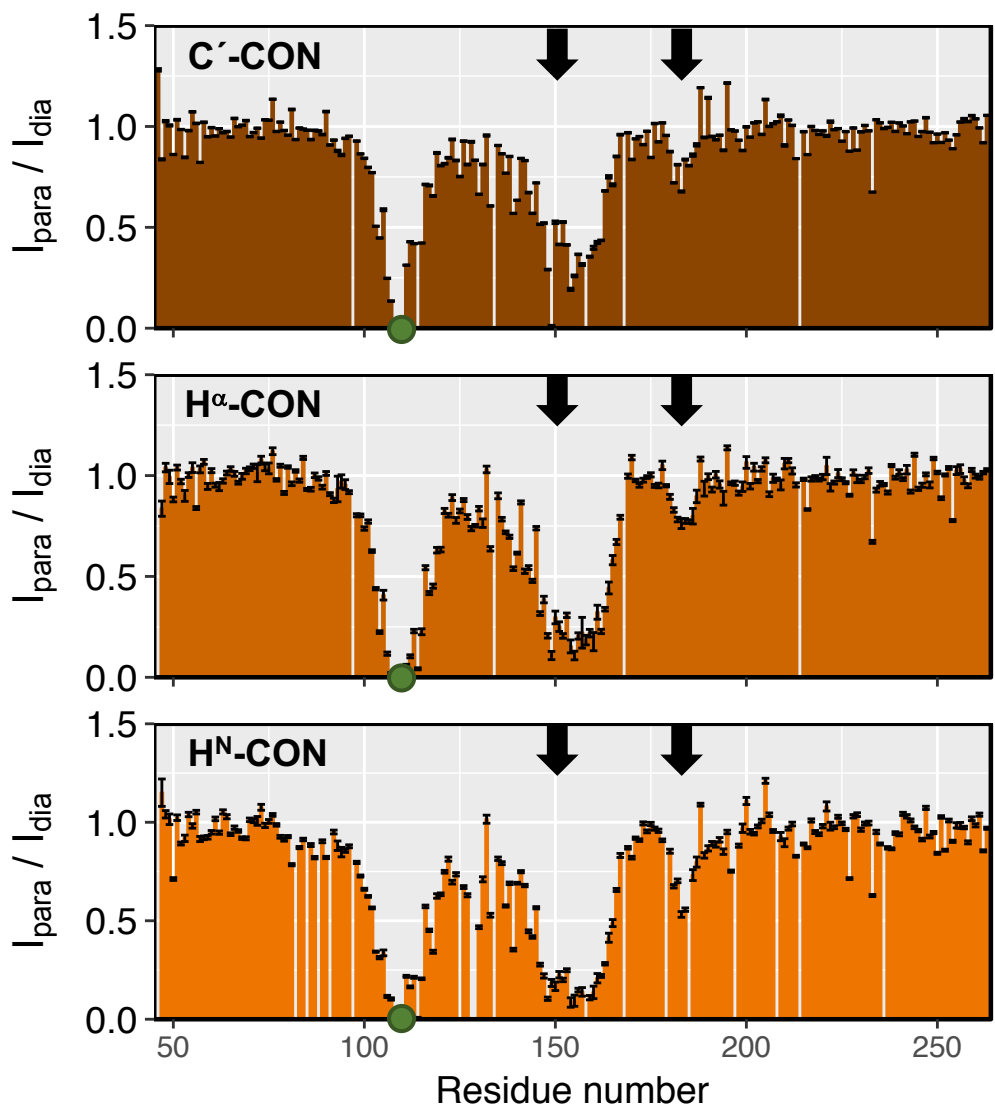
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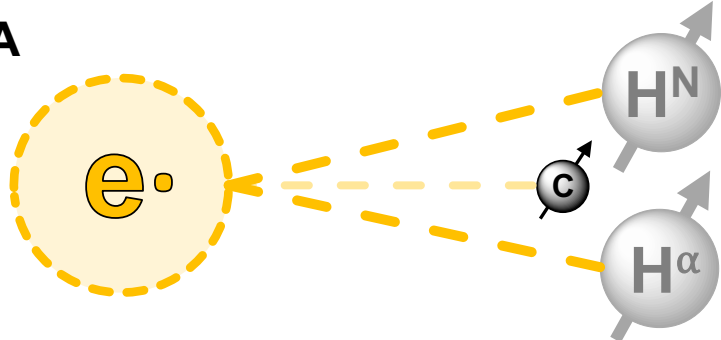
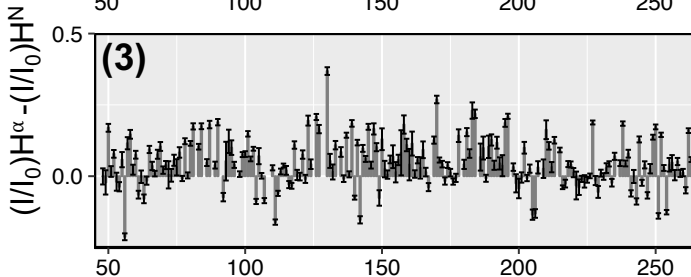
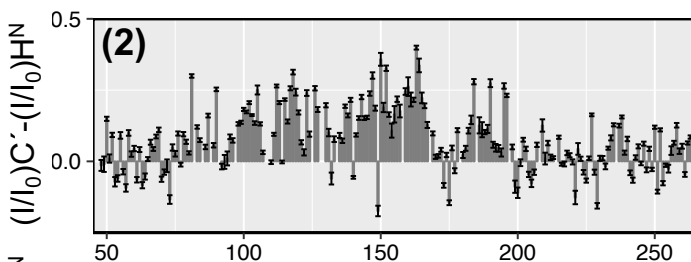
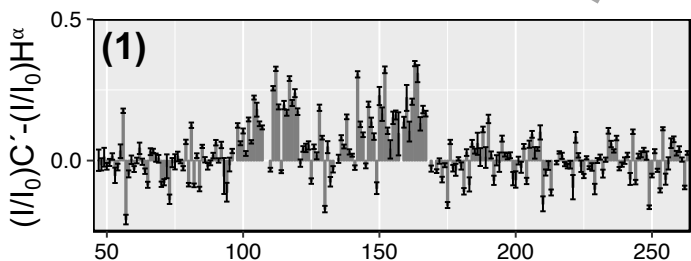
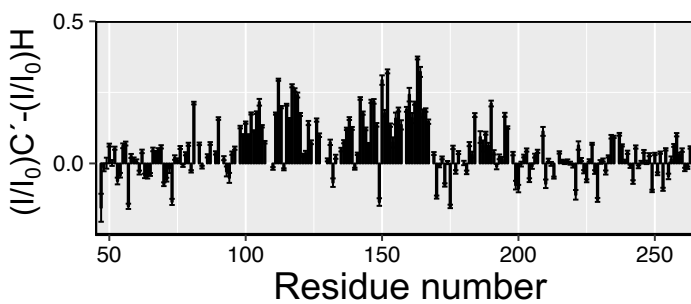


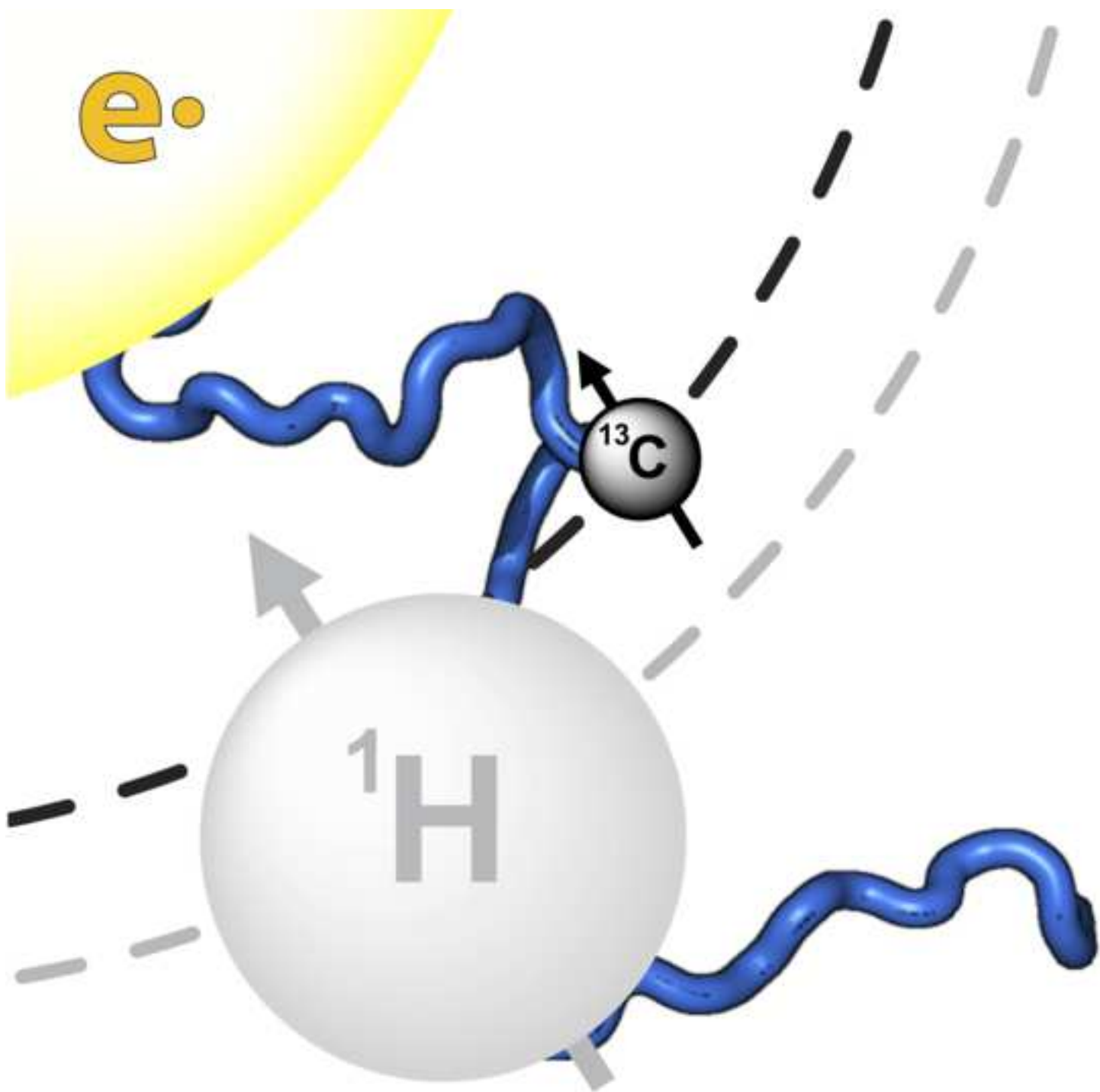
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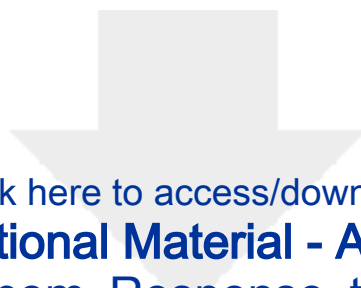






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