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Novel methods based on ^{13}C detection to study intrinsically disordered proteins

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ABSTRACT

Intrinsically disordered proteins (IDPs) are characterized by highly flexible solvent exposed backbones and can sample many different conformations. These properties confer them functional advantages, complementary to those of folded proteins, which need to be characterized to expand our view of how protein structural and dynamic features affect function beyond the static picture of a single well defined 3D structure that has influenced so much our way of thinking.

NMR spectroscopy provides a unique tool for the atomic resolution characterization of highly flexible macromolecules in general and of IDPs in particular. The peculiar properties of IDPs however have profound effects on spectroscopic parameters. It is thus worth thinking about these aspects to make the best use of the great potential of NMR spectroscopy to contribute to this fascinating field of research. In particular, after many years of dealing with exclusively heteronuclear NMR experiments based on ^{13}C direct detection, we would like here to address their relevance when studying IDPs.

1. Introduction

Intrinsically disordered proteins (IDPs) are a class of proteins characterized by the lack of a well-structured three-dimensional fold [1–4]. Despite the absence of a rigid secondary or tertiary structure, IDPs fulfill a number of biological functions, that range from regulation of transcription and translation, storage of small molecules to regulation of self-assembly of large multi-protein complexes [5,6]. The multiple conformations that IDPs can adopt and the presence of highly flexible and solvent exposed backbones turn out to be important for the function of IDPs, conferring them properties complementary to those of structured proteins. For example, IDPs are often able to bind to different partners or to act as hub proteins, playing in this way an important role in a variety of different processes. Bioinformatics predicts that a large portion of the genome encodes for proteins that remain fully or partially disordered in the cell: about one third of the eukaryotic proteins contain in fact unstructured regions that do not possess any regular secondary or tertiary structure. Many examples of functional IDPs are now appearing in the literature at a very fast speed. Therefore, IDPs are now recognized as key players for many different biological functions [7,8]. After initial skepticism, it is now well accepted that the structural and dynamic characteristics of IDPs do confer them functional advantages, demanding the extension of the structure–function paradigm to include a new dimension of high flexibility and disorder.

The general properties of IDPs cannot be captured in ordered crystals, preventing them to be suitable targets for crystallographic studies. Thus, NMR spectroscopy plays a crucial role in the investigation of IDPs, being the only method that allows a high resolution description of their structural and dynamic features in solution [2,3,9–13]. Without the ambition of providing exhaustive answers to all the possible questions, still a variety of different observables can be determined, each of them sensitive to different structural and dynamic properties. Their interpretation provides valuable information on the relative flexibility of different parts of the polypeptide chain and on the sampled conformations. In addition, simple NMR spectra can be used to follow, at residue level, perturbations deriving from changes in the experimental conditions. For example, variations in pH, temperature, ionic strength, and external pressure may be useful to determine protein conformational properties and stability, addition of potential partners enable to monitor interaction sites and binding properties, all valuable information to learn about the functional properties of IDPs.

The properties of IDPs, however, do have an impact on the quality of the NMR spectra (Fig. 1) as well as on the interpretation of the results. Therefore NMR experiments need to be optimized to account for the specific spectroscopic features of IDPs.

The most well-known consequence of the lack of a well-defined 3D structure and of the dynamic inter-conversion between different conformers consists in averaging out the majority of the contributions to chemical shifts arising from the presence of a

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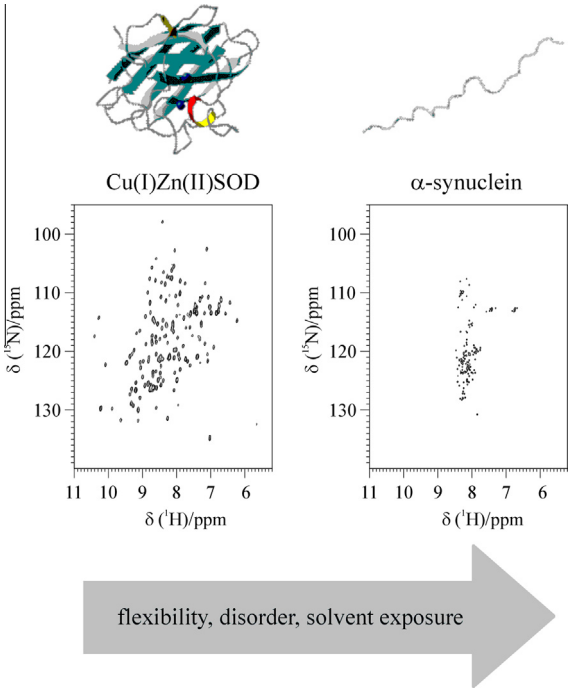

Fig. 1. 2D ^{1}H–^{15}N correlation spectra acquired on two proteins of similar size characterized by very different structural and dynamic properties: a structured one (monomeric Cu(I)Zn(II) superoxide dismutase) and an intrinsically disordered one (α-synuclein). The ribbon of monomeric Cu(I)Zn(II) superoxide dismutase (PDB code: 1mfm) and a schematic representation of an extended backbone are also shown on top of each 2D spectrum to highlight the very different properties of IDPs respect to folded proteins.

structure [14]. This means that the chemical shift ranges are drastically reduced, causing a great degeneracy of signals chemical shifts as evident by comparing the 2D ^{1}H–^{15}N correlation spectra (Fig. 1) acquired on two well-studied proteins, the well-folded human superoxide dismutase (153 amino acids in its monomeric form) and the IDP α-synuclein (140 amino acids), characterized by a similar number of amino acid but by very different structural and dynamic properties. As a consequence, the signal overlap in the spectra of IDPs strongly limits the possibility of standard NMR experiments to provide sufficient resolution for their characterization, especially in the case of amino acid sequences with highly repetitive motifs.

Another factor that should be taken into account is that labile amide protons, which in IDPs are largely exposed to the solvent due to the lack of structural elements, exchange rapidly with water molecules, broadening amide proton resonances beyond detection, with consequent decrease in sensitivity and resolution of the commonly used set of experiments based on amide proton detection. This is particularly true when approaching physiological conditions (neutral pH and body temperature). In addition, IDPs often contain multiple proline-rich polypeptide segments which, lacking the amide protons, cause difficulties in sequential assignment of residues and in accessing high resolution information. All these peculiar properties should thus be taken into account in the design of NMR methods for the characterization of increasingly large and complex IDPs.

In the last years, great improvements in instrumental sensitivity have stimulated the development of a set of exclusively heteronuclear NMR experiments based on ^{13}C direct detection [15]. Thanks to the properties of heteronuclear spins (^{13}C, ^{15}N), these experiments result particularly well suited to study IDPs. The increasing interest that IDPs are attracting has in turn stimulated further improvements, which are here critically discussed, highlighting future perspectives.

2. Practical aspects of ^{13}C detected experiments for biomolecular applications

Before focusing on the performance of ^{13}C detected experiments for the study of IDPs a few general comments on practical aspects are due. The major well known drawback of ^{13}C detection respect to ^{1}H consists in its intrinsically lower sensitivity due to the smaller ^{13}C gyromagnetic ratio respect to that of ^{1}H. Despite this, ^{13}C direct detection is routinely used to study small molecules in solution and to study biosolids. The gain in instrumental sensitivity achieved in recent years has enabled applications of ^{13}C direct detected experiments also to the study of biomolecules in solution. Indeed, in about 10 years, the development of improved probeheads for ^{13}C direct detection with the internal coil dedicated to ^{13}C and the exploitation of the cryo-technology to reduce thermal noise, has brought about an order of magnitude increase in the sensitivity of ^{13}C stimulating progress in this field.

Another aspect to consider when moving to ^{13}C direct detection of uniformly isotopically enriched samples consists in the problem of the large one-bond homonuclear ^{13}C–^{13}C couplings that cause large signal splitting in the direct acquisition dimension. Many solutions have been proposed to address this problem and several approaches were tested [16,17]. As of today, the most widely used experiments to study IDPs are based on direct detection of carbonyl carbons. Among the many methods available to decouple C′ from C^{α}, the most widely used is based on the IPAP approach [18] because of its good performance and easy implementation on any standard NMR equipment. It is interesting to note that once the large homonuclear ^{13}C–^{13}C couplings are suppressed, then the remaining ones are in general smaller than those influencing ^{1}H detection. The various homonuclear decoupling techniques can also be adapted for other types of signals (backbone $^{13}C^{\alpha}$s or specific side chain ^{13}Cs) [17,19].

Finally, an important information to decide whether it is worth to try ^{13}C detected experiments to study a specific IDP consists in having an estimate of the experimental time/sample concentration requirements. A few practical examples on a well-known IDP, human α-synuclein, are probably the best way to address this point. With a sample concentration of about 0.1–0.2 mM, using a 700 MHz NMR instrument equipped with ^{13}C optimized cryogenically-cooled probehead, 2D experiments and the basic 3D CBCACON can be acquired with a few scans per increment. With increasing protein concentration (>0.5 mM), any of the multidimensional NMR experiments based on carbonyl direct detection can be acquired to achieve the complete sequence specific assignment of an IDP using only ^{13}C detected experiments [20–26]. As discussed later in the text, the experimental sensitivity of exclusively heteronuclear experiments based on ^{13}C direct detection can be increased by exploiting ^{1}H polarization as a starting source as well as employing, when appropriate, strategies to reduce the inter-scan delay. Therefore minimal requirements in terms of NMR time or sample concentration can be alleviated.

3. Properties of IDPs and their impact on NMR parameters

3.1. High flexibility and conformational averaging – extensive resonance overlap

The extensive averaging of chemical shifts deriving from the lack of a stable 3D structure is a distinctive feature of IDPs that is generally used to discriminate between well folded and highly flexible, disordered polypeptide chains, as shown in Fig. 1. The