Methodological Advancements for Characterising Protein Side Chains by NMR Spectroscopy

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Keywords: NMR spectroscopy; Method developments; Protein side chains; Conformational Exchange; Interactions.

Short title: Characterising Protein Side Chains by NMR

Abstract

The surface of proteins is covered by side chains of polar amino acids that are imperative for modulating protein functionality through the formation non-covalent intermolecular interactions. However, despite their tremendous importance, the unique structures of protein side chains require tailored approaches for investigation by NMR spectroscopy, and so have traditionally been understudied compared to the protein backbone. Here, we review substantial recent methodological advancements within NMR spectroscopy to address this issue. Specifically, we consider advancements that provide new insight into methyl-bearing side chains, show the potential of using non-natural amino acids, and reveal the actions of charged side chains. Combined, the new methods promise unprecedented characterisations of side chains that will further elucidate protein function.

Revealing the canvas

The chemical structure and conformational sampling of side chains give proteinogenic amino acids their unique characteristics and allow proteins to carry out their biological functions. For example, the polar side chains of proteins form imperative parts of most active sites in enzymes and these cover protein surfaces, as a canvas, and play instrumental roles in substrate recognition and non-covalent interactions. On the other hand, side chains of hydrophobic amino acids form the core of the proteins and are key to protein folding. Nevertheless, most experimental techniques, including nuclear magnetic resonance (NMR) spectroscopy, have traditionally focussed on delineating the dynamics and conformational exchange of the protein backbone. The protein backbone contains one repeating chemical entity, the secondary amide, which has facilitated experimental characterisations. On the other hand, side chains are unique and bespoke methods, each aimed at different chemical motifs and nuclear spin patterns, are required to interrogate them.

The last few years have seen a surge in the availability of NMR methods to characterise the interactions and conformational exchange of proteins side chains (Figure 1). Whereas early methods focussed on methyl groups, exploiting the C_{3v} symmetry to gain insight into large complexes [1–3] as well as characterising the ^{15}N-^{1}H amide-like spin-pair in arginine side chains [4–7], recent methods have been tailored to gain insight on protein side chains, including aromatic and charged groups [8–11].

The sensitive reporters of large macromolecular complexes

Over the last couple of decades, the application of NMR spectroscopy to large proteins and biomolecular complexes with sizes in excess of several hundreds of kilo-Daltons has become routine. This step-change has been primarily facilitated by the development of a suite of methods aimed at methyl groups, the so-called methyl-TROSY technique, with associated advancements in sample preparation and isotope labelling schemes [3,12]. Several recent reviews [13–15] have described the strengths and breadth of methyl-TROSY. We therefore focus primarily on recent advancements made to characterise conformational exchange employing methyl-bearing side chains.

The dynamics and conformational sampling of methyl groups happens on a very broad range of time-scales and a dissection and specific characterisation of the various collective and local motions is of particular interest. Recently methods have been developed that can report on

motions on a very wide time-scale window from *ca.* 5 ms to 0.01 ms exploiting symmetry relations within the methyl group, that is double-quantum *vs.* zero-quantum relaxation [16], or by measuring adiabatic relaxation dispersions [17]. Most applications however are limited in sensitivity to a much narrower range, with different methods available for each time-scale window.

The classical methyl-TROSY Carr-Purcell Meiboom-Gill (CPMG) relaxation dispersion technique [18] reports on the milli-second chemical exchange (5 ms to 0.5 ms) of a multi-quantum coherence (hereafter referred to as ^{13}C MQ CPMG). Although, in most cases, the chemical shift differences between exchanging states, $\Delta\omega$, derived from such experiments predominantly report on the methyl ^{13}C nucleus, significant efforts are continuously being made to allow for the extraction of individual ^{1}H and ^{13}C chemical differences, which report on the structural sampling of the methyl-bearing side chain in question [19–22]. The ^{1}H methyl-TROSY CPMG (^{1}H SQ CPMG) and the ^{1}H triple-quantum relaxation dispersion (^{1}H TQ CPMG) methods [23,24] address this issue and allow a selective determination of ^{1}H chemical shift differences in chemically exchanging systems (Figure 2a). The ^{1}H TQ CPMG experiment effectively triples the ^{1}H $\Delta\omega$ associated with the exchange process making it much more sensitive to exchange processes with small $\Delta\omega$ and can also be adapted to measure the translational diffusion of low-populated states [25]. However, the rapid relaxation of TQ coherences limit the utility of this approach for large systems. On the other hand, the ^{1}H SQ CPMG is less sensitive to small $\Delta\omega$, but exploits favourable relaxation properties of specific transitions making the experiment amenable to characterising very large proteins. An important advantage of ^{1}H CPMG methods in general [23] is that they can reveal exchanging sites with negligible ^{13}C chemical shift differences that would otherwise be missed by the ^{13}C MQ CPMG method alone. Furthermore, a combined analysis of ^{13}C MQ CPMG and the ^{1}H CPMG experiments can provide both ^{1}H and ^{13}C chemical shift differences and a more accurate quantification of the underlying kinetics and thermodynamics of the exchange process.

By developing methods that optimise the selection of slow-relaxing ^{13}C transitions in methyl groups [26], single quantum ^{13}C CPMG relaxation dispersions with improved sensitivity are possible (Figure 2b). Selecting slow-relaxing ^{13}C transitions results in decreased intrinsic relaxation rates in the CPMG relaxation dispersion experiments and thus the ability to quantify the exchange process more accurately. Methods to select specific transitions and manifolds of the

methyl-group $^{13}CH_3$ spin system continue to improve, with recent optimised approaches to select either the spin-1/2 or spin-3/2 manifold [27,28].

Employing the sensitive methyl-reporters in macromolecular complexes using methyl-TROSY has become a versatile and generally accessible technique that is now broadly employed to characterise dynamics and allosteric regulation as well as key biological interactions [29–34].

Carbon detection illuminates side-chain conformations

Traditionally, biomolecular NMR has focussed on the detection of ^{1}H spin-magnetisation because of the high gyromagnetic ratio of the natural abundance ^{1}H compared to ^{13}C and ^{15}N. In some cases, cross-correlated interactions can be exploited to create slow-relaxing transitions and sharp NMR signals as originally employed in the backbone ^{15}N-^{1}H TROSY experiment [35] and later for methyl-TROSY [1] and aromatic side chains [36,37]. However, generally, the large gyromagnetic moment of ^{1}H also leads to faster relaxation of transverse ^{1}H coherences and thus to broader NMR signals compared to ^{15}N and ^{13}C coherences. Furthermore, the chemical shift dispersion is often substantially higher for ^{13}C and ^{15}N nuclei, which has been exploited for both ^{1}H-detected biomolecular NMR spectra and ^{13}C- and ^{15}N-detected backbone experiments [38,39]. Recently it was suggested that ^{13}C-detection could also play an important role for characterising side chains in medium-to-large proteins up to 80 kDa [10]. Using a uniformly [^{2}H,^{13}C] labelled protein sample, well-resolved ^{13}C-^{13}C side-chain correlation maps can be obtained for six protein side chains, including the positively charged arginine and lysine residues (Figure 3). With this method, the high relaxivity of the ^{1}H spin is removed by substituting all proton ^{1}H spins with deuterium, ^{2}H, which has a gyromagnetic ratio approximately 15% of ^{1}H and therefore a relaxivity of approximately 2.3%. The well-resolved ^{13}C-^{13}C side-chain correlation maps that can be obtained using ^{13}C-detection of per-deuterated proteins not only allow a visualisation of the side chain, but also facilitate studies of side-chain conformational sampling. Thus far, it has been shown that ^{13}C chemical exchange saturation transfer (CEST) can be encoded onto the ^{13}C-^{13}C correlation maps to report on millisecond conformational exchange (Figure 3d), and three-bond ^{13}C-^{13}C scalar couplings can be measured to visualise side-chain conformational sampling (Figure 3e). It is envisioned that additional parameters reporting on side-chain conformational exchange and interactions can be obtained employing the ^{13}C-^{13}C method with appropriate additions and adjustments.

Heed the functional parts

Quantifications of the conformational sampling of the aliphatic part of protein side chains, whether from ^{1}H-^{13}C HSQC spectra, methyl-TROSY experiments, or ^{13}C-detected experiments, have provided important insights into the function of proteins and large macromolecular complexes. However, in order to fully appreciate the inner workings of proteins it is necessary to be able to characterise the kinetics and energetics of the individual interactions formed. Such interactions include, but are not limited to, salt-bridges, hydrogen-bonds, cation-π and π-stacking of aromatic rings. Only with methods available to characterise the functional parts of protein side chains, such as aromatic rings and charged groups, will it be possible to fully appreciate these interactions.

Some of the initial experimental characterisations of protein dynamics, which cemented NMR spectroscopy as a technique to probe motions of proteins [40], were focussed on aromatic ring-flips; a 180^{o} rotation about the χ_2 dihedral angle. The dynamics and interactions of aromatic side chains, including aromatic ring-flips, continues to be the focal point of many investigations. Recent studies have shown evidence of concerted ring-flips of tyrosine and phenylalanine side chains in a small protein [41] and identified and quantified hydrogen bonds between tyrosine side chains and DNA phosphate groups [9]. Of particular interest is very recent and seminal work[8] that allows aromatic side chains in proteins of up to ~200 kDa in size to be investigated (Figure 4a). NMR investigations of large proteins typically require spin-states with slow relaxation rates to be created, exemplified by the early backbone ^{15}N-^{1}H TROSY experiment [35] where a coherence with the ^{1}H-^{15}N dipole-dipole interaction counteracting the ^{15}N chemical shift anisotropy is exploited. In this new method [8], an aromatic ^{19}F-^{13}C TROSY effect that leads to a partial cancellation of dipole-dipole and chemical shift anisotropy interactions is exploited to allow for characterisations of ^{19}F-labelled aromatic residues in large proteins. This method also benefits greatly from selective [^{13}C, ^{19}F] labelling techniques that introduce the ^{19}F spin and eliminate one-bond ^{13}C-^{13}C couplings. It is anticipated that ^{19}F labelling of aromatic rings in proteins will provide an important avenue for characterising structure, interactions, and dynamics of proteins [42,43].

The perpetually positively charged side chains of lysine and arginine as well as the histidine imidazole side chain have all attracted substantial attention because of their prominent roles in non-covalent interactions and their high presence within enzymatic sites. Nonetheless, these side chains have traditionally been challenging to characterise at physiological pH and

temperature. Histidine, for example, is often in equilibrium with different protonation states because of its pK_a near neutral pH. Moreover, the presence of χ_1 rotamers and tautomer structures have further hampered detailed characterisations of its interactions and motions. These exchange events have recently been probed using ^{13}C relaxation dispersion [44]. Alternatively, an ^{15}N cross correlated relaxation approach [45], originally developed for the protein backbone and unaffected by micro-millisecond motions [46], can be used to accurately report on pico-nanosecond motions of histidine side chains.

Lysine and arginine side chains are crucial for forming intra- and intermolecular salt bridges. Intramolecular salt bridges often involve acidic protein side chains such as glutamate or aspartate whereas intermolecular salt bridges can involve the negatively charged phosphate of RNA or DNA [47] or acidic side chains of a binding partner. For lysine side chains at low pH, scalar couplings between the lysine amine nitrogen and the DNA phosphate group phosphorous nuclei provide both an indication and quantitative measure of the interaction [48] (Figure 4b). The arginine guanidinium group has, due to its delocalised positive charge, the ability to form a plethora of interactions beyond salt-bridges, including hydrogen bonds, cation-π and π-π interactions [49,50]. Residue-specific qualitative and quantitative characterisations of the interactions formed by arginine guanidinium groups have been challenging mainly, as in the case of histidine, because several micro-millisecond exchange processes have hampered the acquisition of the required NMR spectra. Direct detection of the central guanidinium $^{13}C^{\zeta}$ combined with the observation of multi-quantum coherences created within the guanidinium group have allowed for quantitative measurements of the interactions formed by arginine[11,51]. This includes a determination of the guanidium flip-rate, a 180° rotation about the N^{ε}-C^{ζ} bond, which in turn reports on the strength of interactions formed by the guanidinium group[11] (Figure 4c). However, a full and detailed residue-specific description of the type and strength of interactions formed by arginine guanidinium groups still awaits further methodological developments.

A future in the spotlight

NMR spectroscopy has the potential to provide residue-specific information on the conformational exchange and interactions of macromolecules. An important aspect is that it has recently become possible to obtain more NMR parameters that help to define the role that protein side chains play in protein function. Not only will these advancements assist in unravelling the

mode of catalysis, interaction, and regulation of enzymes and macromolecular complexes, but they might well aid in delineating the important roles that particularly arginine side chains and aromatic residues play in, for example, liquid-liquid phase separation [52,53].

It is anticipated that full descriptions of the mechanisms governed by protein side chains will require a combination of NMR parameters and other experimental measurements or advanced computational techniques. A recent combined analysis of NMR side-chain parameters and cryo-electron microscopy (cryo-EM) densities demonstrated this exciting prospect by providing detailed insight into allosteric regulation of a ClpP1P2 protease [54]. Substantial developments of multi-scale molecular dynamics simulations have now allowed for good agreement with experimentally derived conformational sampling of methyl-bearing side chains [55–57]. Combined with the development of relaxometry NMR [58] to accurately map the motions of protein side chains over a broad range of timescales, the way has now been paved for detailed insight into the conformational sampling, exchange, and interactions of protein side chains. With the recent advancements made, we envisage that the prominent role side chains play in orchestrating protein function will be fully appreciated in the near future.

Conflict of interest statement

Nothing to declare.

Acknowledgements

The authors acknowledge financial supported from the Biotechnology and Biological Sciences Research Council (BBSRC) (refs: BB/T011831/1 and BB/R000255/1).

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- • • of outstanding interest

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Figure legends

Figure 1. Overview of recent methodological advancements in NMR spectroscopy, covered below, highlighted on the structure of the protein Malate Synthase G (PDB: 2JQX).

Figure 2. New advancements made to employ methyl groups to characterise millisecond conformational exchange in large proteins. **a.** A proton ^{1}H single-quantum CPMG relaxation dispersion experiments, which employs an advanced phase cycle ($\psi1$, $\psi2$) of composite pulses. All details provided in ref [23]. **b.** Methyl ^{13}C CPMG relaxation dispersion experiments, including an optimised selection of the slow-relaxing component of single-quantum methyl ^{13}C magnetisation of methyl groups [26]. Having access to both ^{1}H and ^{13}C relaxation dispersions and doing a combined analysis, allows for a more accurate quantification of the conformational dynamics as well as characterisations of more sites.

Figure 3. Characterising protein side chains using ^{13}C-detection and ^{13}C-^{13}C correlation maps. **a**. Side-chain motifs amenable to the method of obtaining ^{13}C-^{13}C correlation spectra (left) and pulse sequence (right). Black boxes are hard non-selective pulses, bell-shaped pulses are frequency selective, $\Delta = 1/(4J_{CC})$ and $T = 1/(2J_{CC})$. All details provided in ref [10]. **b** and **c.** Examples of 2D ^{13}C-^{13}C correlation spectra for proline (**b**) and arginine/lysine (**c**); recorded on the 18 kDa T4 Lysozyme at 278 K. **d.** Example of a chemical exchange saturation transfer encoded in a ^{13}C-^{13}C correlation map, where a 1.15% low populated state is probed in T4 Lysozyme at 278 K. **e.** Example of measuring three-bond ^{13}C-^{13}C scalar couplings by ^{13}C-detection of isoleucine residues in the 80 kDa protein Malate Synthase G at 310 K.

Figure 4. New methods to characterise aromatic and positively charged side chains in proteins. **a.** The discovery of the ^{13}C-^{19}F TROSY effect and specific labelling with ^{19}F in aromatic side chains allows for investigations of aromatic sites in large proteins[8], with substantial improvements compared to ^{13}C-^{1}H spectra. Spectra are from the 42 kDa maltose binding protein at 298 K (reproduced with permission, License number: 5005901382999). **b**. Quantification of ionic interactions and hydrogen bonds involving lysine and tyrosine side chains [9], from measurements of scalar couplings across the non-covalent interaction [47]. **c.** Quantification of the rate of restricted rotation of arginine side chains to report on interactions formed, *e.g.*, side-on salt-bridges, using a multi-quantum chemical exchange (MQ-CEST) approach, demonstrated on the 18 kDa protein T4 Lysozyme at 293 K.