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ARTICLE

The IR-^{15}N-HSQC-AP experiment: a new tool for NMR spectroscopy of paramagnetic molecules

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Abstract A crucial factor for the understanding of structure-function relationships in metalloproteins is the identification of NMR signals from residues surrounding the metal cofactor. When the latter is paramagnetic, the NMR information in the proximity of the metal center may be scarce, because fast nuclear relaxation quenches signal intensity and coherence transfer efficiency. To identify residues at a short distance from a paramagnetic center, we developed a modified version of the ^{15}N-HSQC experiment where (1) an inversion recovery filter is added prior to HSQC, (2) the INEPT period has been optimized according to fast relaxation of interested spins, (3) the inverse INEPT has been eliminated and signals acquired as antiphase doublets. The experiment has been successfully tested on a human [Fe_2S_2] protein which is involved in the biogenesis of iron-sulfur proteins. Thirteen H_N resonances, unobserved with conventional HSQC experiments, could be identified. The structural arrangement of the protein scaffold in the proximity of the Fe/S cluster is fundamental to comprehend the molecular processes responsible for the transfer of Fe/S groups in the iron-sulfur protein assembly machineries.

Keywords Iron-sulfur proteins · Paramagnetic NMR · ^{15}N-HSQC · Pulse sequences · Paramagnetic relaxation · Anamorsin

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Introduction

Solution state and solid state NMR spectroscopy of paramagnetic molecules (Knight et al. 2013; Otting 2010; Arnesano et al. 2006) play a fundamental role in the understanding of many biological processes, such as electron transfer (Ubbink 2012), metal homeostasis (Lutsenko 2010; Banci et al. 2010), metal trafficking (Finney and O'Halloran 2003; Banci et al. 2006; Boal and Rosenzweig 2009; Leary et al. 2009). Metalloproteins offer many examples in which structure, function and dynamics have been found to be redox dependent (Fetrow and Baxter 1999; Bertini et al. 1995; Lyons et al. 1996), or to be driven by the coordination chemistry of the metal center (Caillet-Saguy et al. 2012). Paramagnetic probes (Gaponenko et al. 2004; Keizers and Ubbink 2011; Iwahara et al. 2004) have been used for decades to study structure (Yagi et al. 2013), dynamics and solvent accessibility (Bertini et al. 1997) of large biomolecules (Clore and Iwahara 2009), multidomain proteins (Russo et al. 2013; Bertini et al. 2007) and biomolecular complexes (Volkov et al. 2010), in which only sparse information is available via conventional NMR approaches (Bertini et al. 2002; Balayssac et al. 2006; Machonkin et al. 2002).

The wide, and still growing, range of application of paramagnetic NMR depends on the fact that electronic correlation times of metal centers range from 10^{-13} to 10^{-8} s (Bertini et al. 2001b). As a consequence, nuclear relaxation properties of the environment of the metal centers are versatile. The hyperfine interaction affects NMR shifts and linewidths to different extents and, therefore one faces different scenarios depending on the metal ion, on the coordination environment, and on the nucleus investigated. As summarized in Fig. 1, for a ^{1}H spin at a fixed distance from the metal center, calculated contributions (Bertini

et al. 2001b) to nuclear relaxation and chemical shifts range from null to thousands Hz and hundreds of ppm, depending on the paramagnetic probe.

Large contributions to relaxation and small contributions to chemical shift represent the most challenging situation for resonance assignment. Unfortunately, some of the metal cofactors that play a fundamental role in metalloproteins have negligible magnetic susceptibility anisotropy and, therefore, belong to this category (Bertini et al. 1994b; Abriata et al. 2009). When the magnetic susceptibility tensor of the metal cofactor is not affected by efficient spin-orbit coupling mechanisms, hyperfine shifts only arise from unpaired electron spin density delocalized onto the observed nuclear spin, which typically quenches a few chemical bonds away from the metal center, unless spin polarization mechanisms due to the presence of aromatic ligands occurs (Bertini et al. 1994c). However, the electron spin-nuclear spin dipolar coupling still contributes to nuclear relaxation; as a consequence, signals that do not belong to the first coordination sphere but are spatially close to the metal cofactor, exhibit negligible hyperfine shifts but efficient paramagnetic relaxation enhancements (Im et al. 1998; Skjeldal et al. 1991; Mo et al. 1999).

In iron-sulfur containing proteins (Fe/S proteins, hereafter), relatively long electron relaxation times and a highly symmetric electronic environment around iron ions give rise to efficient nuclear relaxation and negligible hyperfine shifts for resonances of nuclei not belonging to iron bound residues but in spatial proximity to the cluster. The magnetic coupling between iron ions renders Fe/S clusters less paramagnetic than isolated, tetra coordinated iron ions (Wilkens et al. 1998); however the extent of magnetic coupling is difficult to predict "a priori" and may vary even for structurally homologous proteins (Bertini et al. 1992; Banci et al. 1993). An understanding of the structural properties of residues in the proximity of the cluster is crucial for the elucidation of Fe/S proteins structure-function relationships. In the past, this has been successfully addressed for small electron transfer proteins such as High Potential Iron-Sulfur Proteins (HiPIP) and Ferredoxins (Fdx) (Lin et al. 2009; Volkman et al. 1999; Bentrop et al. 1996; Bertini et al. 1994a). Recently, our group faced the problem to characterize the functional properties of Fe/S proteins involved into Fe/S cluster biogenesis (Banci et al. 2011, 2013a; b). At variance with HiPIP and Fdx, characterized by rigid structures and high thermodynamic stability, these proteins show flexible structures and various conformational states, particularly in the proximity of the cofactor binding sites, that are driven by the uptake and release of the Fe/S cluster (Markley et al. 2013; Li et al. 2013). NMR methodologies need to be developed in order to detect signals that, on one hand cannot be identified in 1D NMR experiments because they are overwhelmed from the bulk diamagnetic envelope and, on the other hand, cannot be observed in conventional double and triple resonance experiments because coherence transfer and NOEs are quenched due to fast relaxation. We present here a modified version of the ^{15}N-HSQC experiment which enables the identification of residues at a short distance from the Fe/S cluster and applied it to the largely unstructured, C-terminal domain of the human $[Fe_2S_2]$ protein anamorsin.

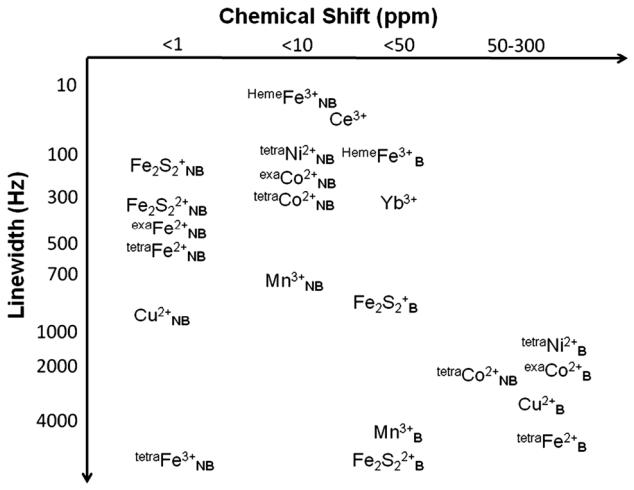

Fig. 1 Expected hyperfine shifts versus expected paramagnetic *line* broadening in ^{1}H resonances for a number of metal centers for directly metal bound (subscript B) and non bound (subscript NB) residues. Tetra and exa *indicate* tetrahedral and octahedral coordination geometries, respectively. Values are estimated by using reported values of electronic relaxation times (Bertini et al. 2001b). For signals from coordinating residues, a 3.5 Å distance from the metal and hyperfine coupling constants ranging 1–4 MHz were considered. For non bound residues, a metal to proton distance of 6 Å was considered. Simulations were performed assuming B_0 = 11.7 T (500 MHz). Scales in both x- and y-axis are arbitrary

Materials and methods

Protein expression and purification

The C-terminal domain of the human protein anamorsin, residues 205-312, named cytokine-induced apoptosis inhibitor 1 (CIAPIN1 domain, hereafter), has been cloned and produced as an independent domain, according to a previously reported methodology (Banci et al. 2013a). This domain was found to bind Fe_2S_2 clusters with two ferric ions ($[Fe_2S_2]^{2+}$, hereafter) (Banci et al. 2011, 2013b).

NMR spectroscopy

Experiments were recorded on a 11.7 T Bruker AVANCE 500 equipped with a triple resonance, inverse detection,

cryoprobe (TXI). All experiments were recorded using the same overall experimental time. Standard ^{15}N-HSQC experiments were recorded using Watergate and flip-back pulses to suppress solvent signal. 96 scans were recorded over 256 increments, with 1.1 s as recycle delay. To collect a fast repetition ^{15}N-HSQC, the GARP4 decoupling scheme was replaced with an adiabatic decoupling that covers 1,500 Hz and uses a 2 ms 180° pulse. 288 scans were recorded over 256 increments with a recycle delay of 350 ms. For IR-^{15}N-HSQC-AP experiments, 2,048 scans were collected over 100 increments (t_{1max}= 24.7 ms), using an INEPT transfer period of 833 us. Acquisition time, recycle delay and inversion recovery (IR) delay were 20, 55 and 50 ms, respectively. T_1 measurements were obtained from a series of ten IR-^{15}N-HSQC-AP experiments recorded using IR delays of 50, 35, 25, 15, 10, 7, 5, 3, 2, 1 ms. Signals characterized by T_1 values longer than 100 ms were measured using a series of experiments using a recycle delay of 350 ms and IR delays of 250, 150, 100, 60, 40, 25, 15, 8, 4 ms.

Results and discussion

The IR-^{15}N-HSQC-AP experiment

The pulse sequence of the IR ^{15}N-HSQC, antiphase detected experiment (IR-^{15}N-HSQC-AP) is reported in Fig. 2. At variance with conventional ^{15}N-HSQC experiments, ^{1}H excitation has been accomplished via an IR building block. IR discriminates nuclear spin magnetizations according to their T_1 relaxation times: a suitable choice of the interpulse delay τ causes a sign discrimination between fast relaxing signals and slow relaxing signals (Gelis et al. 2003; Machonkin et al. 2004). When IR is combined with a short duty cycle, the intensity of diamagnetic signals is significantly suppressed, and the paramagnetic ^{1}H resonances that are usually overwhelmed by the bulk envelope of diamagnetic signals can be observed. In ^{15}N-HSQC experiments, the loss of magnetization during coherence transfer steps is, of course, another factor that modulates signal intensity. Coherence transfer from H_y to $2H_xN_z$ and vice versa competes with relaxation of the $2H_xN_z$ antiphase magnetization which, in a paramagnetic system, is dominated by ^{1}H T_2 relaxation. As shown in Fig. 3, for a ^{1}H signal with T_2 values of 5 ms, about 60% of signal is retained after a standard INEPT transfer step, while only 7% is retained for T_2 values as short as 1 ms. To circumvent the problem, the INEPT period can be shortened according to T_2 relaxation properties of signals of interest (Piccioli and Poggi 2002). When the overall INEPT period is shortened down to 1.5 ms, coherence transfer efficiency of 57, 37 and 17 % are recovered for signals characterized by T_2 values of 5, 2 and 1 ms, respectively, while the efficiency of coherence transfer from nuclei that experience a smaller hyperfine interaction is about 75%. In principle, the hyperfine interaction affects also longitudinal relaxation rates of ^{15}N spins and additional contributions to the relaxation of antiphase H_xN_z magnetization should be considered. However, paramagnetic dipolar relaxation is dependent on the γ^2 of the nucleus and therefore the paramagnetic relaxation experienced by heteronuclear spin can be safely neglected (Bermel et al. 2006). The INEPT approach is useless for ^{1}H resonances with T_2<0.5 ms (Δν ca.> 600 Hz). For the latter case, the coherence transfer pathway never exceeds 10% efficiency and therefore the direct excitation of ^{15}N spins is the best approach (Hsueh et al. 2010).

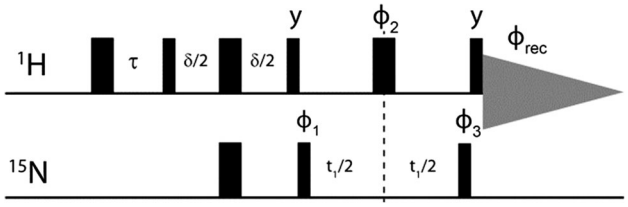

Fig. 2 Pulse scheme of IR-^{15}N-HSQC-AP. Narrow and wide symbols stand for 90° and 180° pulses, respectively. The pulses were applied along the *x* axis unless noted differently. The phase cycling employed is as follows: $\phi_1 = (x,-x)$ $\phi_2 = (x,x,-x,-x)$ $\phi_3 = (x,x,x,x,-x,-x,-x,-x)$ $\phi_{rec} = (x,-x,x,-x,-x,x,-x,x)$. Quadrature in F1 is obtained via States-TPPI of ϕ_1

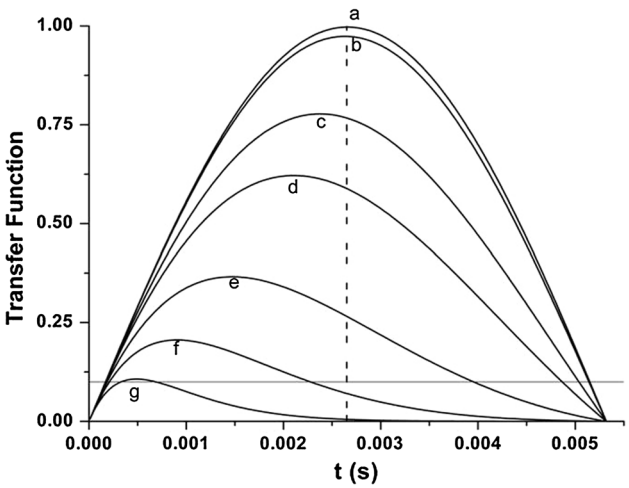

Fig. 3 Efficiency of an INEPT transfer function at different ^{1}H T_2 values: *b* 100 ms, *c* 10 ms, *d* 5 ms, *e* 2 ms, *f* 1 ms, *g* 0.5 ms. Relaxation is neglected in *a*. *Letters* have been drawn at the correspondence of the maximum values for each transfer function. A *dashed line* is shown at the 2.65 ms of INEPT step (94 Hz for ^{1}H-^{15}N J coupling). A *solid line* is shown in correspondence of 10% transfer efficiency

The most critical step of a standard ^{15}N-HSQC experiment is the inverse INEPT block, during which coherences are back converted into observable, in phase Hy. When both forward and backward INEPT steps are used, a signal with $T_2 = 1$ ms ($\Delta\nu$ ca. 300 Hz) will exhibit, under the very best experimental conditions, an overall transfer efficiency of 4%. As a consequence, a reduction of the INEPT period without any pulse sequence modification allows one only to detect H_N connectivities involving ^{1}H resonances $\leq$250 Hz linewidth and fails at larger linewidths. To overcome the problem, we removed the inverse INEPT refocusing step and we acquired the signal as a doublet, antiphase component without ^{15}N decoupling during acquisition. In this simplified scheme, the critical ^{1}H T_2 relaxation is operative only during t_2 acquisition that should be tuned accordingly. The loss in sensitivity arising from the acquisition of the antiphase component versus an in-phase singlet is partly recovered when the direct acquisition dimension is processed with a 90° phase shift. i.e. the antiphase doublet is processed in dispersion mode. When signal linewidth is larger than doublet separation, the components of in phase doublets partly overlap and cancel signal (Bertini et al. 2005). However, when the doublet is phased in dispersion mode, it gives rise to a "pseudosinglet" originated by the sum of the two dispersive components of the doublet. This contributes to the identification of broad, fast relaxing peaks and also to discriminate signals more severely affected by paramagnetic relaxation with respect to the others.

The IR-^{15}N-HSQC-AP experiment is not only useful to identify previously undetected signals, but also provides metal-to proton distances which, in the proximity of the prosthetic group, are the most valuable structural constraint (Bertini et al. 2001a). Indeed, when a series of IR-^{15}N-HSQC-AP experiments is performed in which the interpulse delay of the IR building block, τ, is arrayed, non selective T_1 values of ^1H_N amide resonances are obtained according to a classical IR ^{1}H experiment where the 90° reading pulse is replaced by the ^{15}N-HSQC-AP block. Assuming that ^{1}H relaxation is dominated by the electron spin-nuclear spin metal centered dipolar contribution, T_1 hyperfine relaxation directly depends on r_{MH}^{-6}.

The case of Fe/S proteins

The CIAPIN1 domain of the human protein anamorsin (Banci et al. 2013b; Banci et al. 2011) is a challenging system to test the sequence presented above. It is a 108 amino acid domain that binds a $[Fe_2S_2]^{2+}$ cluster per molecule (Banci et al. 2013a, b). The CIAPIN1 domain is largely unstructured without significant tertiary structure organization (Fig. 4). $[Fe_2S_2]^{2+}$ clusters are expected to induce minimal hyperfine shift and sizable hyperfine nuclear relaxation on ^{1}H spins that do not belong to metal coordinating cysteine residues but are within a, roughly, 10 Å distance from each of the two iron ions. Consistent with expectations, the ^{15}N-HSQC spectrum of the protein, recorded under standard conditions, shows only 71 out of 108 residues. Their assignment was performed via conventional double and triple resonance experiments (Banci et al. 2013a). About 30% of the resonances remain unobserved due to paramagnetic broadening or exchange contributions. As shown in Fig. 4, when IR-^{15}N-HSQC-AP experiment was performed, 10 additional H_N signals, completely absent in previous experiments, are now observed. Furthermore three H_N signals, barely detectable in conventional experiments, significantly increased their intensity. The repetition of the experiment at different IR delays allowed us, from the analysis of integrated intensity of the paramagnetic ^{1}H-^{15}N resonances, to measure the T_1 values for 12 out of 13 paramagnetic ^{1}H signals, that have been identified in IR-^{15}N-HSQC-AP from nuclei spatially close to the $[Fe_2S_2]^{2+}$ cluster. Four of them had T_1 shorter than 10 ms, four in 10–20 ms range, one 20–50 ms range, and three in the 80–150 ms range. An assignment has been proposed for 10 out of 13 resonances by the analysis of chemical shift values, relaxation rates, and ^{13}C-detected experiments tailored to paramagnetic systems (Banci et al. 2013a).

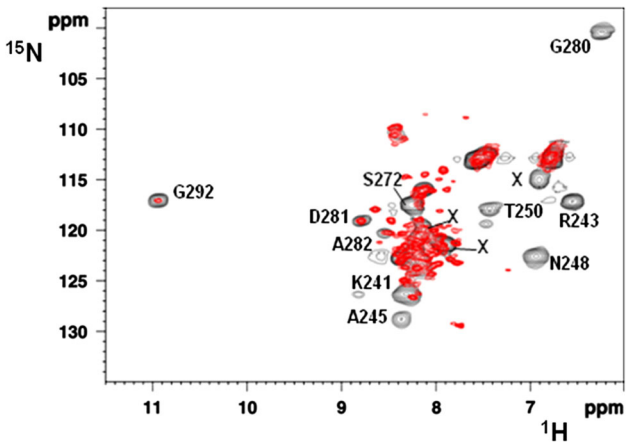

Fig. 4 Overlay of a standard ^{15}N-HSQC (*thin lines*) and IR-^{15}N-HSQC-AP (*thick lines*) experiments acquired on 500 MHz at 298 K on the $[Fe_2S_2]^{2+}$-CIAPIN1 domain. The experimental time for both experiments was ca. 7 h. For the IR-^{15}N-HSQC-AP, acquisition times of 20 and 24 ms were used in t_2 and t_1 acquisition dimensions, respectively, and recycle and IR delays were 55 and 50 ms, respectively. To rule out effects due to short recycle delays, the reference ^{15}N HSQC has been recorded with a 350 ms overall recycle delay. *Peaks* are labeled according to their assignment (Banci et al. 2013a). Unassigned resonances are indicated with X. Signals attributed to D281, A282 and G292 were observed also in the reference experiment but at much lower intensity. For 12 out of 13 *peaks* T_1 values could be obtained. Ten of them have been used as structural constraints

Conclusions

The collection of an exhaustive series of double and triple resonance experiments, in which all parameters have been previously optimized on the basis of reference systems, is nowadays the approach of every NMR laboratory involved in structural biology. Paramagnetic centers cannot be treated with a routine approach and require knowledge of the electronic properties of the metal cofactor of the molecule. We have shown here that fundamental pulse sequence building blocks such as the ^{15}N-HSQC experiment can be easily modified in order to improve the outcome of standard experiments. The IR-^{15}N-HSQC-AP experiment selectively identifies H_N groups from the environment of the paramagnetic center that experience significant paramagnetic relaxation and almost negligible contributions to chemical shift. For the identified H_N resonances, the experiment provides also T_1 relaxation times, which are dependent on the r_{MH}^{-6} on the basis of the Solomon equation (Arnesano et al. 2006) and typically constitute the most precious structural information for metalloprotein structural studies.

This experiment is valuable for those systems in which no pseudocontact shift occurs, such as Cu^{2+} proteins and Fe/S proteins. In particular, we have shown that in the case of proteins involved in the iron-sulfur protein biogenesis, the IR-^{15}N-HSQC-AP experiment substantially contributes to decrease, and eventually abolish, the blind sphere around the metal center that typically escape routine NMR investigation. The structural arrangement of protein scaffold in the proximity of the Fe/S cluster is a fundamental factor to be investigated to comprehend at the molecular level the events responsible of the transfer of Fe/S groups in the iron-sulfur protein assembly machineries.

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