Measuring transverse relaxation in highly paramagnetic systems

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Abstract
The enhancement of nuclear relaxation rates due to the interaction with a paramagnetic center (known as Paramagnetic Relaxation Enhancement) is a powerful source of structural and dynamics information, widely used in structural biology. However, many signals affected by the hyperfine interaction relax faster than the evolution periods of common NMR experiments and therefore they are broadened beyond detection. This gives rise to a so-called blind sphere around the paramagnetic center, which is a major limitation in the use of PREs. Reducing the blind sphere is extremely important in paramagnetic metalloproteins. The identification, characterization, and proper structural restraining of the first coordination sphere of the metal ion(s) and its immediate neighboring regions is key to understand their biological function. The novel HSQC scheme we propose here, that we termed $R_2$-weighted, HSQC-AP, achieves this aim by detecting signals that escaped detection in a conventional HSQC experiment and provides fully reliable $R_2$ values in the range of $^1H R_2$ rates ca. 50–400 s$^{-1}$. Independently on the type of paramagnetic center and on the size of the molecule, this experiment decreases the radius of the blind sphere and increases the number of detectable PREs. Here, we report the validation of this approach for the case of PioC, a small protein containing a high potential 4Fe-4S cluster in the reduced $[\text{Fe}_4\text{S}_4]^{2+}$ form. The blind sphere was contracted to a minimal extent, enabling the measurement of $R_2$ rates for the cluster coordinating residues.

Keywords Paramagnetic NMR · Iron sulfur proteins · Pulse sequences · NMR based structural restraints · Transverse relaxation · Paramagnetic relaxation enhancement

Introduction
The hyperfine interaction between electron and nuclear spins gives rise to additional contributions to chemical shifts and nuclear relaxation, both of which can be used as a source of structural restraints (Piccioli and Turano 2015; Turner et al. 1998). Nowadays, NMR solution structures of paramagnetic macromolecules are obtained by a combination of conventional restraints (Ab et al. 2006; Mori et al. 2008), such as NOE and residual dipolar couplings, and of paramagnetic-based restraints (Arnesano et al. 2006; Clore 2015; Kudhairy, et al. 2020; Parigi et al. 2019; Pintacuda et al. 2007). Depending on which paramagnet is present in the system, different combinations of contact shifts, pseudocontact shifts, paramagnetic relaxation enhancements and cross correlation rates can be used (Koehler and Meiler 2011; Clore and Iwahara 2009; Kateb and Piccioli 2003; Pintacuda 2004). However, since about two decades, many groups have promoted the use of paramagnetism-based NMR restraints to study also diamagnetic proteins: metal binding tags have been used as spin-labels and provide various restraints capable to complement the NMR information available on a native, diamagnetic, derivative (Iwahara et al. 2004; Miao 2019; Matei and Gronenborn 2016; Liu et al. 2014; Nitsche and Otting 2017; Joss and Haussinger 2019). NMR of paramagnetic systems is not anymore a playground reserved to scientists working with inorganic or bio-inorganic systems, but a tool for a larger community of structural biologists with many potential applications (Tang et al. 2006; Softley et al. 2020).
Very recently, we have shown that structural restraints derived from Paramagnetic Relaxation Enhancements (PRE) can be used as the unique source of restraints for the structure calculation of small metalloproteins (Trindade et al. 2020b). A key point for a successful PRE-only approach is the availability of many accurate relaxation rate values measured throughout the entire protein, including the close proximity of the paramagnetic center, where nuclear spins are strongly affected by paramagnetism. When hyperfine shifted signals are well isolated outside the bulk diamagnetic envelope, they can be characterized using 1D experiments and relaxation based filters (Inubushi and Becker 1983), although usually only a few signals, arising from the side chains of metal-bound residues, can be identified via this approach (Hansen and Led 2006; Sato et al. 2003; Brancaccio 2017). Paramagnetic relaxation depends on $\gamma^2$ of the observed nucleus, therefore $^{13}$C or $^{15}$N direct detection in conventional HSQC experiments. For longitudinal $^1$H relaxation rates, we have shown that an inversion recovery filtered $^{15}$N HSQC experiment acquired in antiphase ($^{15}$N IR-HSQC-AP) (Ciofi-Baffoni et al. 2014) is very effective for the identification of signals severely affected by paramagnetism, and also for obtaining $R_1$ rates faster than those measurable in established experiments. However, it has been reported that $^1$H $R_2$ values are less susceptible to internal motions and cross relaxation than $^1$H $R_1$ values (Iwahara et al. 2004; Iwahara and Clore 2010). Therefore, we are interested to develop an experiment to obtain accurate measurements of $^1$H transverse relaxation also in the close proximity of a paramagnetic center.

The experiment we present here has been customized for the case of the HiPIP (High Potential Iron-sulfur Protein) PioC from Rhodopseudomonas palustris TIE-1 (Bird, et al. 2014). PioC has 54 amino acids, contains the typical HiPIP binding motif CXXX$_n$CX$_m$C and it is the smallest HiPIP isolated so far. Due to the high reduction potential ($E^0 = +450$ mV vs SHE) of the [Fe$_4$S$_4$$]^{3+}$/[Fe$_4$S$_4$$]^{2+}$ redox pair (both oxidation states are paramagnetic), the protein is stable in the reduced [Fe$_4$S$_4$$]^{2+}$ state. The paramagnetic $^1$H NMR spectrum of PioC is very similar to the NMR spectra of other HiPIPs in the reduced state (Bertini 1992), thus indicating that the electronic relaxation time ($\tau_e$) in PioC must be similar to previously studied HiPIPs (Banci et al. 2018). However, PioC is the smallest HiPIP isolated so far and about 60% of the protein is affected by paramagnetic relaxation. Counter-intuitively, the small size of the protein makes it more difficult to study, because paramagnetic effects are active in the majority of the protein, scalar and dipolar couplings are quenched and a number of HN signals are not observable in a conventional HSQC experiment (Cheng and Markley 1995; Machonkin et al. 2005; Lin 2009). Throughout this article we will first review why experimental approaches that are effective to study proteins containing metal bindings tags are not equally efficient for native metalloproteins; then we will discuss how implementation of this novel HSQC scheme allows the measurement of relaxation rate values in a range 50–400 s$^{-1}$. Rates in this range were once very difficult to measure reliably, however they are necessary to reduce the blind sphere enabling the characterization, and proper structural restraining of the first coordination sphere of the metal ion(s) and its immediate surrounding residues.

**Materials and methods**

**Protein expression and purification**

PioC was expressed and purified as previously reported (Bird et al. 2014). Uniformly $^{15}$N labeled samples of PioC were produced and the expression and purification protocol was identical throughout except in the addition of ammonium sulfate ($^{15}$N$_2$, 99%) in the M9 minimal media. BL21 DE3 cells were double transformed with pET32h, a plasmid containing the construct thioredoxin–6xHis–thrombin cleavage site–PioC, and with pDB1281, a plasmid that carries the machinery for the assembly of iron-sulfur clusters. Cells were grown in Luria–Bertani (LB) medium supplemented with 100 mg*dm$^{-3}$ ampicillin and 35 mg*dm$^{-3}$ chloramphenicol until the OD$_{600nm}$ of 1 and then harvested and washed in M9 minimal media salts before resuspension in M9 minimal media. Once re-suspended, cells were incubated for one hour before induction with 0.5 mM IPTG. After 4 h cells were harvested by centrifugation and disrupted using a French Press at 1000 psi. The lysate was ultra-centrifuged at 204,709×g for 90 min at 4 °C to remove cell membranes and debris and the
supernatant was dialyzed overnight against 50 mM potassium phosphate buffer pH 5.8 with 300 mM NaCl before injection in a His-trap affinity column (GE Healthcare). The fraction containing Histag-PioC eluted with 250 mM imidazole and was incubated overnight with Thrombin (GE Healthcare) for digestion. The final purified PioC (His-tag free) was then concentrated from the flow through of a 2nd passage through the His-trap column using an Amicon Ultra Centrifugal Filter (Millipore) with a 3 kDa cutoff. The purity of PioC was confirmed by SDS-PAGE with Blue Safe staining (NzyTech) and by UV–Visible spectroscopy.

NMR spectroscopy

Measurements of $^1$H $R_2$ transverse relaxation rates were carried out using 11.7 T Bruker AVANCE 500 equipped with a triple resonance, inverse detection, cryoprobe (TXI). $^1$H $R_2$ measurements were obtained from a series of $R_2$-weighted $^{15}$N-HSQC-AP experiments, developed throughout this article. For each experiment, 256 scans were collected over 256 increments. Acquisition time and recycle delay were 47.1 ms and 150 ms. A series of sixteen $R_2$-weighted $^{15}$N-HSQC-AP experiments was recorded, using INEPT transfer periods of 0.2 ms, 0.4 ms, 0.6 ms, 0.8 ms, 1.2 ms, 1.6 ms, 2.0 ms, 2.4 ms, 2.8 ms, 3.2 ms, 4.0 ms, 4.8 ms, 5.6 ms, 6.4 ms, 8.0 ms, 10.0 ms. Total experimental time was about 58 h. $^1$H $R_2$ measurements have been obtained also with the established approach (Donaldson 2001), in which a relaxation delay $T$ is inserted into the INEPT building block of an in-phase $^{15}$N HSQC experiment. For each experiment, 32 scans were collected over 156 increments, using acquisition time and recycle delay of 47.1 ms and 4 s, respectively. A series of fourteen $^{15}$N-HSQC-IP experiments was recorded using relaxation delays $T$ of 13.3 ms, 17.3 ms, 21.3 ms, 33.3 ms, 45.3 ms, 57.3 ms, 69.3 ms, 81.3 ms, 93.3 ms, 117.3 ms, 141.3 ms, 165.3 ms, 205.3 ms and 245.3 ms. A 1400 μs selective $^1$H$_z$ inversion pulse was used for $^3$J$_{HNH_α}$ decoupling. Total experimental time was about 80 h. Both series have been performed using 256 × 1024 data point matrices, over spectral windows of 80.0 ppm × 21.7 ppm. Squared cosine weighting functions and apodization were used in both dimensions prior to FT, spectra dimensions was 512 × 2048 data points. Peak intensities were used to calculate $R_2$ values, using the equations described in the results section. All relaxation data were analyzed using the Bruker Topspin Dynamics Center.

NMR assignment of PioC

The backbone NMR assignment of PioC (Trindade et al. 2020a) has been published on Biomolecular NMR Assignment and deposited in the BMRB data bank (ID 34487).

Results

State of the art: an overview

The state-of-the-art approach for $^1$H $R_2$ relaxation is to use a $^{15}$N HSQC experiment and insert a relaxation period within the INEPT block to measure $H_N$ rates (Donaldson 2001). Relaxation rates $R_2$ can then be obtained either by collecting, for each $H_N$ signal, a complete decay curve or by measuring $R_2$ rates from a two time-point measurement, thereby enabling the direct determination of $R_2$ values and their associated errors without any fitting procedure (Iwahara et al. 2007). For non-deuterated proteins, band selective $^1$H 180° pulses should be used to avoid the evolution of $^3$J$_{HNH_α}$ coupling during the relaxation period. The paramagnetic contribution to the overall nuclear relaxation is given (Bertini et al. 2016) by Eq. (1)

$$R_{2obs} = R_{2para} + R_{2dia}$$ (1)

when $R_{2dia}$ and $R_{2obs}$ values can be measured with high precision and accuracy (Iwahara 2007), then very small values of paramagnetic relaxation enhancement $R_{2para}$ can be obtained by the difference of the two measured values. This has been exploited by attaching a metal binding peptide, such as ATCUN or HHP at the N-terminus site of a diamagnetic protein, as shown in Fig. 1a (Donaldson 2001). Metal binding peptides containing ions with long electronic correlation times, such as, for instance, Cu$^{2+}$ or Mn$^{2+}$, provide paramagnetic relaxation enhancements that can be measured, in the case of Cu$^{2+}$ ion, for all protons located approximately 10–30 Å apart from the metal center (Donaldson 2001; Harford and Sarkar 1997; Jensen et al. 2004; Keizers and Ubbink 2011). The strength of this approach is that, within the above range, both $R_{2obs}$ and $R_{2dia}$ can be experimentally measured with high accuracy and $R_{2para}$ as small as 0.5 s$^{-1}$ can be obtained, thus allowing the use of Paramagnetic Relaxation Enhancement (PRE, hereafter) for long metal-to-proton distance restraints. Protein–protein interaction surfaces and catalytic centers typically fall within this range, therefore one could obtain additional structural information for protein–protein/protein–ligand interactions, structure refinement, etc. (Battiste and Wagner 2000; Hass and Ubbink 2014; Cetiner 2019; Anthis and Clore 2015; Sprok 2018). On the other hand, signals at less than 12 Å from the copper(II) ion will experience $R_{2para}$ larger than 50 s$^{-1}$, that will give rise to signals that, in the $R_2$ experiment, will be very weak and eventually broadened beyond detection. The loss of information typically occurs in a protein region where no catalytic reactions or biochemical events occur, since the metal binding peptide is attached far from the protein core, usually at the N-term site. In metalloproteins, however, the topology of the system
is completely different: the metal center(s) and its first coordination sphere always constitute the core region for the protein function. Structural biologists are therefore interested to obtain detailed information in the close proximity of the metal center(s), where the biochemically relevant events occur. Assuming the same paramagnetic relaxation enhancement of the previously described situation, we face a loss of information in the most interesting part of the protein. Such blind region (Balayssac et al. 2006) is, indeed, always the core region of a metalloprotein (Fig. 1B). In this frame, reducing the blind sphere around the paramagnetic center(s) by measuring relaxation rates of nuclear spins that are most affected by paramagnetism becomes extremely important.

The replacement of Cu(II), i.e. the metal ion used in the case of Ubiquitin shown in Fig. 1a, with another metal ion will change the radius of the sphere where paramagnetic relaxation enhancement is effective and measurable, but the above consideration will remain: the metal center is always surrounded by a blind sphere, where signals are broadened beyond detection, and by an outer sphere, in which PREs can be measured and factorized. The situation is described in Fig. 1c, where we considered 50 s\(^{-1}\) and 0.5 s\(^{-1}\) as upper and lower limits for the detection of PREs, the simulated behavior of paramagnetic centers with different \(\tau_e\) is shown.
spin \((S = 1/2)\) Fe\(^{3+}\), with \(\tau_e = 1 \times 10^{-12}\) s, they would be 4 Å and 9 Å, respectively. Different electronic relaxation times, together with other experimental parameters, such as magnetic field strength and protein size will give rise to different radii for the blind sphere and for the sphere where PREs are measurable. Also the replacement of \(^1H\) with \(^{13}C\) or \(^{15}N\) nuclei reduces both spheres and provides an alternate route to obtain PREs (Mateos et al. 2019). To extend the range of application of PREs in \(^1H\) detected experiments it is necessary, on the one hand, to increase the accuracy of experimental methods and observe smaller PRE values and, on the other hand, to measure with reliability \(R_2\) values larger than those achievable with the existing experimental approaches.

As extensively studied in the past, \([Fe_4S_4]^{2+}\) clusters in proteins have an electronic ground state \(S = 0\) due to the antiferromagnetic coupling (Phillips et al. 1970; Mouesca and Lamotte 1998; Bertini et al. 1992). However, each iron ion is formally in the oxidation state \(Fe^{2.5+}\) and the system is paramagnetic at room temperature due to the population of the excited energy levels of the electron spin ladder (Banci et al. 1990). The distribution of population among the spin levels depends on the extent of the antiferromagnetic coupling constant(s) operative in the \([Fe_4S_4]^{2+}\) cluster (Blondin and Girerd 1990). Therefore, the choice of the parameters to input in the Solomon equation to predict the behavior of \(R_2\) vs H-Fe distance in \([Fe_4S_4]^{2+}\) clusters is not obvious (Bertini et al. 1997): if we consider that each iron ion will relax with the same electronic relaxation time of a High Spin \(Fe^{2+}\) ion \((\tau_e = 5 \times 10^{-12})\), and that paramagnetism at room temperature arises from the population of the first excited state of the electron spin energy ladder, characterized by \(S = 1\), we obtain, as shown in Fig. 1C, a behavior somehow intermediate between the two cases considered here for iron ions, with expected values for the blind sphere and for the PRE sphere of about 6 Å and 13 Å. This makes PioC an ideal test case for the optimization of sequences aiming at measuring \(R_2\) rates in the proximity of the cluster.

### Pulse sequence description

Here we propose a novel pulse sequence, shown in Fig. 2a, in which the relaxation delay is embedded within the INEPT evolution, the refocusing INEPT is removed and signal is acquired as antiphase doublet as soon as the \(H_N\) magnetization is created by the last \(^1H\) 90° pulse. We called the experiment \(^1H\) \(R_2\)-weighted \(^{15}N\)-HSQC-AP because the INEPT period, typically \(1/(2J_{HN})\), is replaced here by a variable relaxation delay \(T\). In this very simple sequence, \(^1H\) transverse relaxation is active only during the delay \(T\), when the \(2H_N\) coherence evolves from \(H_\alpha\) as \(H_N\sin(\pi J_{HN} T)\). The relaxation rate of the \(2H_N\) antiphase term is a combination of \(^{15}N\ \tau_1\) and \(^1H\ \tau_2\) relaxation rates; paramagnetic relaxation rates have a \(\gamma^2\) dependence from the observed nucleus (Solomon 1955; Bertini 1986), therefore the contribution of \(^{15}N\ \tau_1\) can be neglected and the observed rates are fully due to \(^1H\ \tau_2\) relaxation (Iwahara et al. 2007). Also cross correlation between \(^1H\) Curie Spin Relaxation and HN dipole–dipole relaxation is not contributing to the observed rates (Pintacuda et al. 2003; Mori et al. 2010). In order to sample \(^1H\) relaxation rates also at \(T\) values of a few μs, we avoid using pulsed field gradients, generally applied during the INEPT period. The delay \(T\) can then be arrayed from zero to 10 ms to sample the evolution of \(H_\alpha - 2H_N\) coherence transfer for half a sinusoidal period \((1/J_\alpha)\), during which \(^1H\ \tau_2\) relaxation is active. To avoid signal losses due to \(^1H\ \tau_2\) relaxation, the inverse INEPT block is removed and the \(2H_N\) coherence, created by the two 90° pulses at the end of \(^{15}N\) evolution, is acquired in antiphase without \(^{15}N\) decoupling. Removing the \(^{15}N\) decoupling also rules out duty cycle problems: without decoupling, one can safely use very short recycle delays in order to increase S/N of fast relaxing signals and to suppress water signal via progressive saturation (Camponeschi et al. 2019). This may affect the observed relaxation rates of \(H_N\) signals; however, when the observed values in Eq. (1) are dominated by \(R_{2para}\) water saturation should not play a significant role. Another very important feature of this sequence is the suitability for cryo-probes, because there are no risks associated to coil heating due to an excess of RF power (Helms and Satterlee 2013). The use of a refocusing INEPT and \(^{15}N\) decoupling during acquisition is indeed a severe limiting factor for the recycle delay that, by no means, could have been as short as we used in our experiments.

The \(R_2\)-weighted HSQC-AP is essentially the simplest possible scheme for measuring \(^1H\ \tau_2\) relaxation with an HSQC-type experiment. Figure 2b shows the features of the \(R_2\)-weighted building block with respect to the relaxation building block commonly used (Donaldson 2001). For the latter, the relaxation delay \(T\) must accommodate the selective \(^1H\) 180° pulse, the Pulsed Field Gradients, the INEPT transfer period \(2\tau_x\). The shortest possible value of the relaxation delay \(T\) is set to about 12 ms, indeed this is why very fast relaxing signals are not observed with this approach. The removal of the \(^1H\) 180° selective pulse and shorter gradients allow one to decrease this value, even though it can’t be below 6.5 ms. As shown in Fig. 2b, for an \(R_2\) rate of 70 s\(^{-1}\) signals will be already at 50% of the initial intensity at the first time point of the series, while those exceeding 150 s\(^{-1}\) will be beyond detection after two time points of the \(R_2\) series, for all these situations the exponential decay could not be properly analyzed. On the other hand, the \(R_2\)-weighted INEPT building block is highly complementary to this approach, because it will monitor the evolution of signal intensities during the first 10 ms of the relaxation recovery, starting from \(T\) period as small as 100 μs, therefore being able to monitor also very fast relaxing signals. Another
interesting feature of the $R_2$-weighted HSQC is that fast and slow relaxing resonances will be measured with comparable precision.

As expected, the most representative spectra of the two $R_2$ series are rather different. In Fig. 3a, the first time point of the exponential decay of the in-phase experiment is superimposed to the spectrum of the $R_2$-weighted HSQC-AP series, recorded with $T = 2.4$ ms. Several signals, such as those of residues 22, 27–28, 36–37, 47–51, are indeed observable only in the $R_2$-weighted HSQC-AP, while other residues are only barely visible in the first point of the exponential decay (e.g. residues 25 and 35). Fast relaxing

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**Fig. 2** The $^1$H $R_2$ weighted $^{15}$N HSQC-AP pulse experiment. a Pulse sequence used for the experiment. Hard 90° and 180° pulses are used for both $^1$H and $^{15}$N channels, using phase x when undefined. Phase cycling: $\phi_1 = x, -x, y, -y$; $\phi_2 = 2(y), 2(x); \phi_3 = 2(x), 2(-x); \phi_4 = 4(x), 4(-x)$; $\phi_{rec} = x, -x, x, -x$. PFG gradients of 200 ms were used, with 100 ms for gradient recovery. Standard parameters to measure fast relaxing nuclei as follows: $aq = 47$ ms, $T = $ ranging from 200 ms to 10 ms, recycle delay $= 150$ ms. b Comparison between different $R_2$ relaxation building blocks. Considering the relaxation building block shown on the left panel (Donaldson 2001; Iwahara et al. 2007), using $\tau_a = 2.65$ ms and $T/4 = 1.8$ ms as shortest possible delay, the exponential decay can be measured from about 12.5 ms. Signals with $R_2$ values over 70 s$^{-1}$ will be lost or detected at very weak intensities for few points. In the $R_2$-weighted building block shown in the right panel, they could be easily measured for a sufficient number of time delays in the range 0–10 ms, provided signals have enough S/N. The time scales of the two curves highlight the complementarity of the two experiments.
signals are much better observed in the $R_2$ weighted experiment, although signals are much broader than in the in-phase experiment, because they have been acquired as HN antiphase doublets. Like homonuclear $^1$H and $^{13}$C cases, a dispersion phase mode of the antiphase doublets produces the sum of the two dispersive components of opposite phase, thus giving rise to a pseudo-singlet with the maximum of signal intensity (Turner 1993; Bertini et al. 1994, 2005a).

As an example, Fig. 3b shows selected rows for His7 and Asn27. In the case of His7, which has a negligible $R_{\text{para}}$ contribution, we observe that, when the signal linewidth is smaller than the splitting of the HN doublet, the typical pattern of an antiphase doublet phased in dispersion mode is observed; when signals are broader than the scalar coupling constant, like the case of Asn27 ($R_2 = 221$ s$^{-1}$), the doublet splitting is lost and the dispersive component of the doublet give rise to a well pseudo observable pseudo singlet. The removal of the inverse INEPT prevents transverse relaxation to be operative before $^1$H detection. Indeed, an additional refocusing would prevent the observation of signals characterized by $T_2$ shorter than the INEPT period.

Fitting of $R_2$ values. The intensity of observed signals in the $R_2$-weighted HSQC-AP can be analyzed according to:

$$I(t) = I_0 \sin(pJt) \exp(-TR_2)$$

where $T$ is the variable delay. In principle, a three-parameter fitting will provide values for $I_0$, $J$ and $R_2$, respectively representing the initial signal intensity, the $^1$J$_{\text{HN}}$ scalar coupling constant, and the $^1$H $R_2$ relaxation rate. However, the interplay between the two parameters $J$ and $R_2$ is such that a three parameters fitting of the buildup curves tends either to over-estimate $J$ and then compensate it with an under-evaluation of $R_2$ values or the other way around (smaller $J$ and higher $R_3$ values). We therefore used a two-parameter fitting for Eq. (2), with constant $J$ values. As shown in Fig. 4a, for $J$ values in the range 92–96 Hz, i.e. the range of admissible values for $^1$J$_{\text{HN}}$ scalar coupling at 500 MHz (residual dipolar couplings are expected to be negligible at 500 MHz for a protein of this size containing a $[\text{Fe}_4\text{S}_4]^{2+}$ cluster), the deviations among calculated $R_2$ values were much smaller than the errors observed in each two-parameters fitting. We therefore decided to consider values and uncertainties taken from the two-parameter fitting obtained using $J = 94$ Hz as a fixed value. Some representative build-up curves are reported in Fig. 4b. The $R_2$-weighted $^1$H-HSQC-AP experiment is able to measure $R_2$ values for all HN signals of the protein, including those belonging to residues of the iron-bound cysteines, to residues H-bonded to cluster sulfide ions, and those spatially close to the 4Fe-4S cluster although not in direct electronic contact with the prosthetic group.

**Data evaluation and assessment**

The $R_2$ values obtained with the $R_2$-weighted HSQC-AP experiments are summarized in Table 1, together with those measured using the standard sequence. A data assessment is reported in Fig. 5, where, for both series, the value of the relative error ($\Delta R_2/R_2$) vs $R_2$ is shown. When $R_2$ values are lower than 45 s$^{-1}$, the accuracy of the sequence based on exponential decay and in-phase acquisition is much higher than that of the $R_2$-weighted-HSQC-AP. Indeed, when INEPT ($2\tau_a$) and $R_2$ relaxation ($T$) evolve into separate building blocks, a single exponential decay is measured and several methods allow one to measure $R_2$ values with very good precision and accuracy (Iwahara et al. 2007). However, in the range 45–80 s$^{-1}$ the relative errors become larger than those...
obtained with the $R_2$-weighted-AP and, above 80 s$^{-1}$, $R_2$ values are measurable only (with the experimental conditions we used to perform the experiments) with the $R_2$-weighted-AP sequence, up to $R_2$ rates as large as 310 s$^{-1}$. This indicates that, for signals with $R_2$ rates larger than ca. 50 s$^{-1}$, the $R_2$-weighted-AP sequence should be the preferred method to measure relaxation rates, while the standard approach should be used in all other cases. Because all backbone HN signals of PioC have been identified and assigned and this was the fastest rate among them, it is not possible to set the upper limit threshold of the $R_2$-weighted-HSQC-AP experiment. In principle (see Fig. 2b), one should be able to measure $R_2$ values up to 400–500 s$^{-1}$, provided that very broad signals do not fall within a crowded spectral region.

The poor agreement between the two sets of values obtained with the two experiments and reported in Table 1 also deserves a comment. Indeed, the $R_2$-weighted HSQC-AP experiment has been optimized to measure $R_2$ rates of fast relaxing signals, which are the target of this experiment. To this end, we have used, for the $R_2$-weighted HSQC-AP, recycle delays that are a factor 20 shorter than those used in the in-phase sequence (200 ms vs 4 s). Due to the fast repetition of the experiment, signals with $R_2 < 35$ s$^{-1}$ suffer from partial saturation. As a matter of speculation, it would always be possible to perform the $R_2$-weighted HSQC-AP experiment with longer recycle delays to properly fit $R_2$ values of slower relaxing signals. However, the obvious complementarity between the two experiments, given by the time scale of the recoveries measurable with the two experiments, and the intrinsic higher precision of the in phase experiment provided by the single exponential dependence, suggest that a combination of two $R_2$ measurements, respectively optimized for the quantification of small and large PREs would be, by far, the most efficient approach.

**Conclusions**

In summary, the $R_2$-weighted HSQC-AP experiment, proposed here, is the simplest experiment for $R_2$ relaxation, in which $^1$H magnetization is kept along the transverse plane only during the relaxation delay and $t_2$ acquisition and all periods of $J_{HN}$ evolution/refocusing are removed. This simplified scheme not only detects signals that escape detection in a conventional HSQC experiment, but measures $R_2$ values that are fully reliable, in the range of rates 50–400 s$^{-1}$. This is extremely important for metalloproteins, where the first coordination sphere of the metal ion(s) and its immediate...
Table 1 500 MHz, 298 K, observed transverse relaxation rates for PioC HN amide protons

| δ\(^1\)H (ppm) | δ\(^15\)N (ppm) | residue | R\(_2\) (s\(^{-1}\)) | error (s\(^{-1}\)) | R\(_2\) (s\(^{-1}\)) | error (s\(^{-1}\)) |
|---------------|-----------------|---------|-------------------|-----------------|-------------------|-----------------|
| 8.26          | 117.2           | THR     | 2                 | 11.1            | 0.4              |
| 8.42          | 123.7           | LYS     | 3                 | 15.4            | 1.3              |
| 8.43          | 121.8           | LYS     | 4                 | 23.0            | 6.9              | 19.1            | 1.5              |
| 8.76          | 115.2           | SER     | 6                 | 38.8            | 5.2              | 17.8            | 0.6              |
| 9.08          | 116.3           | HIS     | 7                 | 37.8            | 4.4              | 21.3            | 0.8              |
| 8.49          | 116.5           | LYS     | 8                 | 52.9            | 19.1             | 32.8            | 2.2              |
| 8.13          | 119.5           | ASP     | 9                 | 35.3            | 5.3              | 16.9            | 0.7              |
| 8.23          | 118.2           | ALA     | 10                | 34.6            | 4.3              | 19.6            | 0.8              |
| 7.69          | 105.0           | GLY     | 11                | 46.8            | 3.9              | 20.0            | 1.4              |
| 8.19          | 116.3           | TYR     | 12                | 36.6            | 3.8              | 29.9            | 1.5              |
| 8.67          | 124.5           | GLN     | 13                | 54.3            | 3.8              | 46.4            | 4.1              |
| 8.22          | 115.2           | GLU     | 14                |                 |                  | 19.4            | 1.1              |
| 7.29          | 112.8           | SER     | 15                | 21.9            | 5.8              | 21.6            | 1.0              |
| 7.92          | 116.4           | ASN     | 17                | 31.2            | 4.4              | 22.8            | 1.1              |
| 8.17          | 111.6           | GLY     | 18                | 43.5            | 5.2              | 25.1            | 1.5              |
| 8.78          | 126.9           | ALA     | 19                | 52.6            | 12.0             | 44.4            | 2.8              |
| 7.62          | 118.4           | LYS     | 20                | 41.8            | 10.9             | 17.8            | 0.8              |
| 7.47          | 115.9           | ARG     | 21                | 38.0            | 2.8              | 44.4            | 3.1              |
| 9.47          | 131.4           | CYS     | 22                | 313.6           | 19.6             | n.o             |
| 8.62          | 97.5            | GLY     | 23                | 60.3            | 6.2              | 48.8            | 6.3              |
| 8.62          | 109.8           | THR     | 24                | 45.9            | 4.1              | 42.0            | 2.8              |
| 8.36          | 130.0           | CYS     | 25                | 65.0            | 5.1              | 44.1            | 7.5              |
| 9.28          | 131.4           | ARG     | 26                | 47.9            | 4.9              | 55.5            | 5.3              |
| 9.62          | 141.7           | GLN     | 27                | 221.0           | 15.1             | n.o             |
| 7.72          | 123.4           | PHE     | 28                | 172.3           | 8.4              | n.o             |
| 8.19          | 128.2           | ARG     | 29                | 39.2            | 5.2              | 44.9            | 3.0              |
| 8.15          | 111.8           | SER     | 32                | 43.5            | 5.2              | 25.1            | 1.5              |
| 7.55          | 114.9           | SER     | 33                | 43.4            | 4.3              | 24.2            | 0.8              |
| 8.55          | 129.0           | CYS     | 34                | 37.5            | 3.9              | 42.5            | 3.7              |
| 8.36          | 126.0           | ILE     | 35                | 84.5            | 5.9              | 65.6            | 26               |
| 8.75          | 127.6           | THR     | 36                | 115.0           | 12.3             | n.o             |
| 7.00          | 149.9           | VAL     | 37                | 161.5           | 10.7             | n.o             |
| 8.61          | 122.8           | GLU     | 38                | 43.0            | 4.0              | 24.2            | 0.8              |
| 8.43          | 115.7           | SER     | 39                | 35.2            | 8.3              | 19.7            | 1.0              |
| 7.28          | 118.2           | ILE     | 41                | 25.4            | 7.5              | 27.7            | 1.3              |
| 7.84          | 116.4           | SER     | 42                | 36.7            | 3.8              | 42.8            | 2.4              |
| 9.12          | 121.2           | GLU     | 43                | 24.1            | 3.4              | 19.2            | 0.5              |
| 7.98          | 116.8           | ASN     | 44                | 25.7            | 5.2              | 27.4            | 1.5              |
| 7.20          | 102.6           | GLY     | 45                | 55.5            | 3.8              | 46.2            | 6.5              |
| 7.86          | 113.7           | TRP     | 46                | 73.1            | 5.0              | 54.8            | 14.8             |
| 6.47          | 118.9           | CYS     | 47                | 182.7           | 11.0             | n.o             |
| 6.79          | 115.2           | ARG     | 48                | 57.9            | 5.3              | 74.0            | 28.6             |
| 7.74          | 154.7           | LEU     | 49                | 168.4           | 12.4             | n.o             |
| 6.02          | 119.5           | TYR     | 50                | 56.6            | 14.3             | n.o             |
| 5.46          | 119.0           | ALA     | 51                | 216.6           | 42.0             | n.o             |
| 8.33          | 109.7           | GLY     | 52                | 39.4            | 4.4              | 23.0            | 1.3              |
| 8.43          | 120.2           | LYS     | 53                | 24.9            | 5.0              | 18.0            | 0.9              |
| 8.04          | 128.5           | ALA     | 54                |                 |                  | 7.7             | 0.3              |
| 8.53          | 124.0           | TRP sc  | 46                | 53.1            | 4.2              | 33.9            | 2.1              |

Columns 5–6 refer to values observed with the R\(_2\)-weighted HSQC-AP experiment, columns 7–8 to values observed with an in-phase HSQC experiment with a single exponential T\(_2\) decay prior to INEPT.
were 4 s and 200 ms, respectively. For $R^2$ values under 40 s$^{-1}$, data from the in-phase experiment have much smaller errors than those from the $R^2$-weighted HSQC-AP experiment. For $R^2$ values over 50 s$^{-1}$, the $R^2$-weighted-HSQC-AP has much better performances. For $R^2$ values above 80 s$^{-1}$, only the $R^2$-weighted-HSQC-AP provided reliable $R^2$ measurements.

Fig. 5 Transverse relaxation rates percentage errors ($ΔR^2/R^2$) obtained with the two series of experiments discussed in Fig. 2: (red) in-phase detected HSQC with relaxation delay preceding INEPT; (blue) $R^2$-weighted HSQC-AP. The recycle delays for the two series were 4 s and 200 ms, respectively. For $R^2$ values under 40 s$^{-1}$, data from the in-phase experiment have much smaller errors than those from the $R^2$-weighted HSQC-AP experiment. For $R^2$ values over 50 s$^{-1}$, the $R^2$-weighted-HSQC-AP has much better performances. For $R^2$ values above 80 s$^{-1}$, only the $R^2$-weighted-HSQC-AP provided reliable $R^2$ measurements.

neighboring regions can be identified, characterized and, finally, restrained into NMR structure calculations only when it is possible to obtain paramagnetism-based structural restraints such as PREs. The small HiPIP protein PioC is a challenging and significant example of the application of this experiment, where this objective was achieved and the $R^2$ could be measured for all four cysteines coordinating the paramagnetic cluster. Finally, it is worth noting that this approach can be useful in all other cases where $R^2$ is larger than 50 s$^{-1}$: conformation dynamics and exchange phenomena often increase relaxation rates above this threshold also in diamagnetic systems, thus extending the potential applications of this approach.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Availability of data and material: Complete protein NMR Assignment reported in BMRB ID-34487. Pulse sequences and NMR data sets available upon request.

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