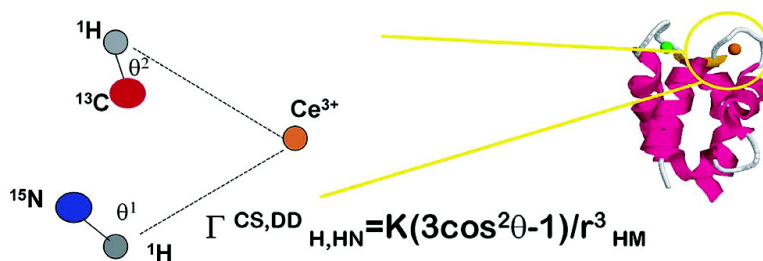


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## New Routes to the Detection of Relaxation Allowed Coherence Transfer in Paramagnetic Molecules

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The use of paramagnetic centers, either native or artificial, to assess structural and dynamic properties of biomolecules is quite fashionable in contemporary biomolecular NMR.<sup>1</sup> Within the quest for paramagnetism-based NMR structural constraints,<sup>2</sup> cross correlation rates between <sup>1</sup>H Curie spin relaxation<sup>3</sup> and <sup>1</sup>H–X (X = <sup>1</sup>H, <sup>15</sup>N, or <sup>13</sup>C) dipole–dipole coupling ( $\Gamma_{\text{H,HX}}^{\text{Curie,DD}}$  hereafter) are a powerful source of information, to date largely unexploited.

Discovered since a decade<sup>4</sup> and, since then, studied by several groups,<sup>5–6</sup> they have been used, in the case of cross correlation rates between <sup>1</sup>H<sub>N</sub> Curie spin and HN dipole–dipole coupling, as long-range constraints to refine macromolecular structures<sup>7</sup> and to obtain structures without NOEs.<sup>8</sup> They have been proven to be strongly synergistic to residual dipolar couplings and pseudocontact shifts, and they have been already implemented within the most common programs for structure calculations<sup>9</sup> due to their distance and angular dependence shown in eq 1

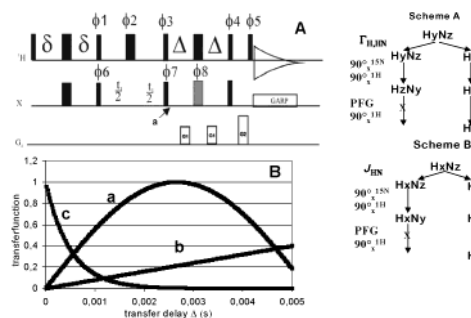
$$\Gamma_{\text{H,HN}}^{\text{Curie,DD}} = \frac{K}{r_{\text{MH}}^3} (3 \cos^2 \theta - 1) \quad (1)$$

where M refers to the metal ion,  $\theta$  is the angle between the M–H and H–N (or H–C) vectors, and  $K$  depends on the magnetic field and on the electron spin  $S$ .<sup>10</sup>  $\Gamma_{\text{H,HN}}^{\text{Curie,DD}}$  behave as the well-known diamagnetic cross correlation rates between <sup>1</sup>H chemical shift anisotropy (CSA) and H–N dipole coupling ( $\Gamma_{\text{H,HN}}^{\text{CSA,DD}}$ )<sup>11</sup> such that, in paramagnetic molecules, the experimentally observable quantity  $\Gamma_{\text{H,HN}}$  is given by<sup>7,9</sup>

$$\Gamma_{\text{H,HN}} = \Gamma_{\text{H,HN}}^{\text{CSA,DD}} + \Gamma_{\text{H,HN}}^{\text{Curie,DD}} \quad (2)$$

Until now  $\Gamma_{\text{H,HN}}^{\text{Curie,DD}}$  values in paramagnetic systems have been collected using the same pulse sequences<sup>7,12</sup> designed to detect  $\Gamma_{\text{H,HN}}^{\text{CSA,DD}}$ . When these sequences are applied to paramagnetic systems, fast transverse self-relaxation quenches the build-up of relaxation allowed coherence transfer<sup>13</sup> (RACT) and prevents the detection of coherences arising from fast relaxing <sup>1</sup>H signals close to the paramagnetic center.<sup>7,9,14</sup> There is therefore the need to develop alternative routes to measure  $\Gamma_{\text{H,HX}}$  in which RACT could be detected before being quenched by self-relaxation. We present here a modified version of an HSQC experiment, designed to observe and quantify cross correlation rates in those cases in which standard pulse sequences fail to detect a RACT peak.

When XH groups in spatial proximity of a paramagnetic center are studied via a conventional HSQC experiment, the scalar coupling coherence transfer (SCCT hereafter) from H to X and vice versa is quenched because of paramagnetic induced <sup>1</sup>H transverse relaxation.<sup>15</sup> To overcome this problem, transfer delays in HSQC experiments tailored to paramagnetic signals are not optimized to the maximum of SCCT function.<sup>16</sup> Indeed, the H<sub>y</sub>X<sub>z</sub> (X being <sup>13</sup>C or <sup>15</sup>N) coherence has to evolve only for very short delays to avoid the loss of information due to <sup>1</sup>H self-relaxation.<sup>6</sup>



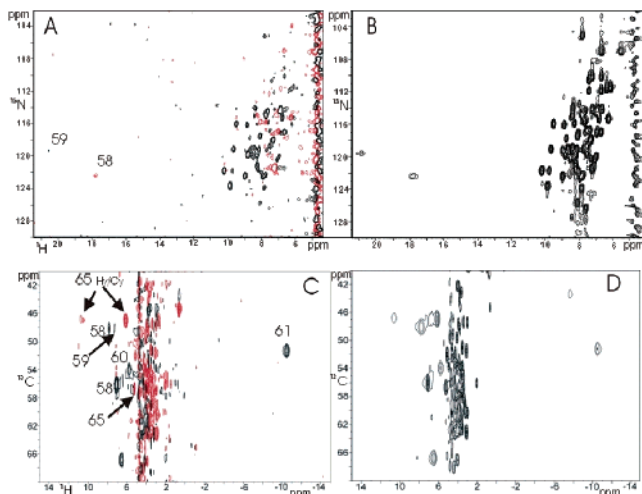
**Figure 1.** Pulse scheme for quantitative  $\Gamma_{\text{H,HX}}$  measurements. The gray pulse is applied only in the reference experiment (Scheme B). All pulses have phase  $x$ , unless indicated. Phases:  $\phi 1 = y$ ;  $\phi 2 = 2x, 2(-x)$ ;  $\phi 3$  (scheme A) =  $16x, 16y, 16(-x), 16(-y)$ ;  $\phi 3$  (scheme B) =  $16y, 16(-x), 16(-y), 16x$ ;  $\phi 4 = 16(-x), 16y, 16x, 16(-y)$ ;  $\phi 5 = -x$ ;  $\phi 6 = x, -x$ ;  $\phi 7 = 4x, 4(-x)$ ;  $\phi 8 = 8x, 8(-x)$ ; receiver =  $2(x, -x), 2(-x, x)$ . (B) Coherence transfer functions due to: (a) scalar coupling coherence transfer, (b) relaxation allowed coherence transfer and (c) self-relaxation decay as a function of the transfer delay  $\Delta$ . Self-relaxation equally affects both SCCT and RACT. Simulation curves were obtained with  $\lambda = 1000 \text{ s}^{-1}$ ,  $\eta = 20 \text{ s}^{-1}$ ,  $J = 94 \text{ Hz}$ .<sup>19</sup> The evolution of coherence transfer in the two schemes from point a is also shown.

The same principle could be used when the coherence transfer occurs through relaxation interference. The scheme is reported in Figure 1A. Because RACT is expected to be less efficient than SCCT, we exploit scalar coupling for the H–X transfer, and we allow cross correlation to evolve only in the back transfer step from X to H via a CRIPT-type building block<sup>17,18</sup> during which H<sub>y</sub>N<sub>z</sub> is converted to H<sub>y</sub> via RACT. Prior to detection, a  $z$  filter is added to select only signals arising from the wanted coherence transfer. A 90° change in the phase of the first proton pulse of the X–H coherence transfer ( $\phi 3$ , in Figure 1A) and the insertion of a 180° refocusing <sup>15</sup>N pulse (gray-colored, in Figure 1A) will transform the RACT building block in a trivial reverse INEPT step, thus allowing, via a transfer H<sub>x</sub>N<sub>z</sub> → H<sub>y</sub>, to acquire a reference spectrum for quantitative analysis of RACT. The use of a  $J$  coupling evolution period for referencing the cross-correlated transfer is, to our knowledge, unprecedented in the literature.

As long as the evolution delay  $2\Delta$  is shorter than  $1/2J$ , the time dependence of both SCCT and RACT is linear (Figure 1B).<sup>19</sup> Self-relaxation will equally affect the time dependence of SCCT and RACT.<sup>19</sup> As a consequence, the ratio between intensities collected in a RACT experiment and in a SCCT experiment will be independent from the evolution delay  $\Delta$  and related to the absolute value of  $\Gamma$  (expressed in hertz) according to eq 3

$$I_{\text{RACT}}/I_{\text{SCCT}} = -\Gamma/J \quad (3)$$

On this basis, two experiments, performed with very short  $\Delta$  delays, provide a direct measurement of  $\Gamma_{\text{H,HN}}^{\text{Curie,DD}}$  even for those peaks which are strongly affected by the paramagnetic center.



**Figure 2.** 700 MHz  $^1\text{H}$ - $^{15}\text{N}$  (A) and  $^1\text{H}$ - $^{13}\text{C}$  (C) RACT spectra of CaCeCb, obtained with the proposed sequence and corresponding reference spectra (B) and (D). All peaks which are strongly affected by the hyperfine interaction but are still observable in the HSQC experiments, shown a peak in the RACT experiments. The experiments have been carried out, using a Bruker 700 MHz Avance Spectrometer, on a 1.2 mM protein sample, at unbuffered pH = 6 at 300 K. For  $^1\text{H}$ - $^{13}\text{C}$  experiments delays durations were: recycle delay = 450 ms,  $\delta$  = 800  $\mu\text{s}$ ,  $\Delta$  = 1.2 ms. For  $^1\text{H}$ - $^{15}\text{N}$  experiments delays were: recycle delay = 450 ms,  $\delta$  = 1 ms,  $\Delta$  = 1 ms. Experimental conditions are reported in ref 22. In both cases experiments were performed using  $\Delta$  values from 0.8 to 1.2 ms.

Because eq 3 does not depend on  $\Delta$ , different choices for  $\Delta$  allows to assess the accuracy of the approach.

Test experiments are reported for the  $^{13}\text{C}$ ,  $^{15}\text{N}$ -labeled di-calcium binding protein calbindin  $\text{D}_{9k}$ <sup>20</sup> in which a  $\text{Ca}^{2+}$  ion is replaced by  $\text{Ce}^{3+}$  (CaCeCb). As previously shown,<sup>21</sup> amide groups of residues Asp 58 and Gly 59 are paradigmatic cases of HN pairs strongly affected by the hyperfine interaction. Indeed, peaks corresponding to Asp 58 and Gly 59, which were completely unobserved when measuring cross correlation rates using the standard sequence<sup>9</sup> are now detected in the RACT experiment, as shown in Figure 2, A and B. Overall, 63  $\Gamma_{\text{H,HN}}$  values were obtained (Table S1). They are in a very good agreement with  $\Gamma_{\text{H,HN}}$  values obtained using standard pulse sequences,<sup>12</sup> even for those signals for which  $\Gamma_{\text{H,HN}}$  values arise entirely from  $\Gamma_{\text{H,HN}}^{\text{CSA,DD}}$  (Figure S1).

This method can be fruitfully extended to cross correlation rates involving  $^1\text{H}$  Curie spin relaxation and C-H dipole-dipole coupling ( $\Gamma_{\text{H,HC}}^{\text{Curie,DD}}$ ).  $\Gamma_{\text{H,HC}}$  involving H CSA and C-H dipole-dipole coupling have never been reported. Unlike  $\Gamma_{\text{H,HN}}^{\text{CSA,DD}}$ , they are independent of secondary structure, thus being useless in terms of structure validation and analysis.<sup>23</sup> The scenario becomes interesting when a paramagnetic center induces substantial amounts of Curie spin relaxation onto the  $\text{H}\alpha$  proton. Then,  $\Gamma_{\text{H}\alpha,\text{H}\alpha\text{C}\alpha}$  in the proximity of the metal center are dominated by the  $\Gamma_{\text{H}\alpha,\text{H}\alpha\text{C}\alpha}^{\text{Curie,DD}}$  contribution. As in the previous case, RACT is detected during the very short transfer delays that are mandatory in paramagnetic systems, as shown in Figure 2, C and D. All signals that are affected by the hyperfine interaction are clearly observed in the  $^{13}\text{C}$ - $^1\text{H}$  RACT spectrum. Using our experiments, 54 H/CH cross correlation rates could be obtained (Table S2), many of them being part of the first coordination sphere of  $\text{Ce}^{3+}$ .

In conclusion, we have shown that the proposed sequence is unique in providing quantitative estimate of  $\Gamma_{\text{H,HN}}$  and  $\Gamma_{\text{H,HC}}$  in the close proximity of the metal center. This is crucial to refine the immediate proximity of the metal ion. At variance with pseudo-contact shifts<sup>24</sup> and residual dipolar couplings,<sup>25</sup> conversion of  $\Gamma$  values into geometrical constraints is not mediated from the

calculation of the anisotropy tensor of the molecule. As a consequence, even a single  $\Gamma$  value can be converted into a structural constraint without any loss in reliability.

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**Supporting Information Available:** Tables of  $\Gamma_{\text{H,HN}}$  and  $\Gamma_{\text{H,HC}}$  values; comparison with data obtained with standard sequences; comparison between experimental  $\Gamma$  values and values back-calculated on the basis of NMR solution structure (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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