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Geometry dependent two-dimensional heteronuclear multiplet effects in paramagnetic proteins

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Abstract

We report experimental observation and numerical simulation of a two-dimensional multiplet effect in the heteronuclear correlation spectrum of a paramagnetic protein that depends on molecular geometry. This effect arises as a consequence of cross-correlated relaxation involving the Curie spin relaxation and internuclear dipolar relaxation mechanisms. It also manifests itself in resolution and sensitivity improvement in transverse relaxation optimised spectroscopy (TROSY) kind of experiments. Characteristic multiplet patterns in heteronuclear coupled two-dimensional NMR spectra encode directional information for the heteronuclear bond with respect to the paramagnetic center. These patterns, which are simulated here using Redfield's relaxation theory, can be used to obtain a new type of geometry restriction for structure determination and refinement of paramagnetic macromolecular systems.

Introduction

Electron paramagnetism is a widely known cause of line broadening in high resolution NMR spectra. While for diamagnetic target molecules established sample preparation protocols usually avoid or remove any paramagnetic components, relaxation enhancement by electron paramagnetism has also found useful applications to accelerate experiments or to derive distance information (Kopple and Zhu, 1983; Petros et al., 1990; Esposito et al., 1992). The importance of transverse relaxation interference effects, which in general are manifest as differential broadening in multiplets, and prevail conspicuously at higher magnetic field strengths, can be ascribed to the following two facts. Firstly, the sensitivity and resolution enhancement as provided by the TROSY experiment have boosted the size limit of proteins that are amenable to structural analysis by NMR to above 30 kDa (Salzmann et al., 1998). Secondly, the geometry dependence of cross-correlated relaxation can be exploited to obtain long-range structural constraints (Boisbouvier et al., 1999; Kloiber and Konrat, 2000).

Here we report theoretical results and experimental evidence showing that under suitable conditions a two-dimensional (2D) differential line broadening effect occurs in coupled heteronuclear spectra of paramagnetic proteins. This happens by virtue of cross correlated relaxation involving the Curie spin relaxation (CSR) mechanism due to the magnetic polarisation of a paramagnetic centre (Gueron, 1975; Vega and Fiat, 1976). Some aspects of cross correlation effects of CSR have been examined in detail previously (Bertini et al., 1993; Mäler et al., 1995; Ghose and Prestegard, 1997; Desvaux and Gochin, 1999). In a paramagnetic protein the cross correlation phenomena involve three important relaxation mechanisms, namely, dipolar (DD), chemical shift anisotropy (CSA) and CSR. As will be shown by simulations, their mutual interference can give rise to unique multiplet patterns in 2D NMR spectra, which carry information on the relative

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positions of spin pairs with respect to a paramagnetic centre. We have experimentally observed such 2D multiplet patterns in ^{1}H-^{15}N spin pairs of ^{15}N labelled wild type sperm whale cyanometmyoglobin (electron spin $S_e = 1/2$) at a magnetic field strength of 11.7 T. The experiments confirm the theoretical simulations and exhibit a noticeable line narrowing for any of the four components of the 2D ^{1}H-^{15}N cross peaks in heteronuclear single quantum correlation spectra [HSQC (Palmer et al., 1991; Schleucher et al., 1994)] without spin decoupling. The remaining three multiplet components are broadened to different extents. In the ^{1}H and ^{15}N coupled 2D HSQC spectrum of cyanometmyoglobin, Figure 1, one can distinguish cross peak multiplet patterns belonging to four different classes, according to the intensity ratios of the four multiplet components.

In diamagnetic proteins only the multiplet pattern shown in Figure 1b can be found. This known multiplet effect is the basis of a series of seminal papers by Wüthrich and co-workers that introduced the principle of TROSY (Pervushin et al., 1997; Salzmann et al., 1998; Riek et al., 1999), which, through sensitivity and resolution enhancement greatly expands the molecular size range of macromolecular NMR. Three additional types of cross peak patterns observed in a paramagnetic protein arise from cross correlation phenomena involving CSR, as will be outlined below. These 2D patterns are a consequence of a flip in the proton doublet intensities, experimentally first observed for certain residues close to the electron centre in cytochrome (Ohmura et al., 1998), and an analogous effect for the ^{15}N doublet.

TROSY-type experiments exploit differential line narrowing due to cross correlation phenomena among the DD and CSA relaxation mechanisms (DD×CSA) for resolution and sensitivity enhancements at higher fields. In the case of paramagnetic proteins, the additionally present paramagnetic relaxation gives rise to several contributions (Solomon and Bloembergen, 1956; Gueron, 1975; Vega and Fiat, 1976; Kowalewski, 1996), one of which is CSR. Of all the contributions from paramagnetic relaxation only CSR has non-negligible cross correlation spectral density with other relaxation mechanisms under the conditions that will be detailed below. Formally, the spin dynamics of CSR is similar to that of CSA (Ghose and Prestegard, 1997). In the case of DD×CSA cross-correlations the principle axes of the interaction tensors span a very narrow angular range around 0°, for bound *NH* and *CH* pairs. In contrast, the angles between the DD and CSR tensors can take any value depending on the relative orientation of the heteronuclear bond vector with respect to the paramagnetic centre. Therefore the geometry has a major influence on the spectral densities and consequently on the magnitude and character of the differential narrowing effect.

Previous research efforts have already underscored the potential of CSR towards refinement of structures of paramagnetic proteins, firstly, by observing ^{1}H relaxation rates of backbone residues (Boisbouvier et al., 1999) and secondly, by investigating its influence on the ^{1}H relaxation patterns of methyl groups (Mandal et al., 2000).

The plots in Figure 1b–e clearly show different line narrowing patterns in coupled HSQC, which points to the benefits of using TROSY-kind experiments even at 500 MHz on such a paramagnetic protein sample. Resolution and in some cases sensitivity enhancements as compared to conventional (decoupled) HSQC can hence be expected when selecting only the narrowest multiplet component. We emphasise here again that, in contrast to the case of diamagnetic proteins, the enhancement can occur in any of the four components, requiring more spin state selective experiments (e.g. Andersson et al., 1998; Weigelt, 1998) to benefit from enhancement in each of the components.

The cross peak patterns convey inherent geometry information. To illustrate this we have simulated multiplets of coupled ^{1}H-^{15}N systems assuming different orientations of the ^{1}H-^{15}N bond with respect to an unpaired electron centre by solving the time evolution of the single quantum coherences of both ^{1}H (I) and ^{15}N (S) spins in the framework of Redfield's relaxation theory (Redfield, 1965). In the following we give explicit expressions for the transverse relaxation rates of the S spin, those for the I spin can be obtained by interchanging the indices S and I. The time dependence of the S spin single quantum coherences can be obtained from the expectation values of the single transition operator equation as (Goldman, 1984):

$$\frac{d}{dt}\begin{pmatrix} S_+^{(1)}(t) \\ S_+^{(2)}(t) \end{pmatrix} = -\begin{pmatrix} R_{1212} + i(\omega_S + \pi J_{IS}) & R_{1234} \\ R_{3412} & R_{3434} + i(\omega_S - \pi J_{IS}) \end{pmatrix} \begin{pmatrix} S_+^{(1)}(t) \\ S_+^{(2)}(t) \end{pmatrix} \quad (1)$$

where ω_S is the Larmor frequency of the S spin and J_{IS} the scalar coupling constant between the I

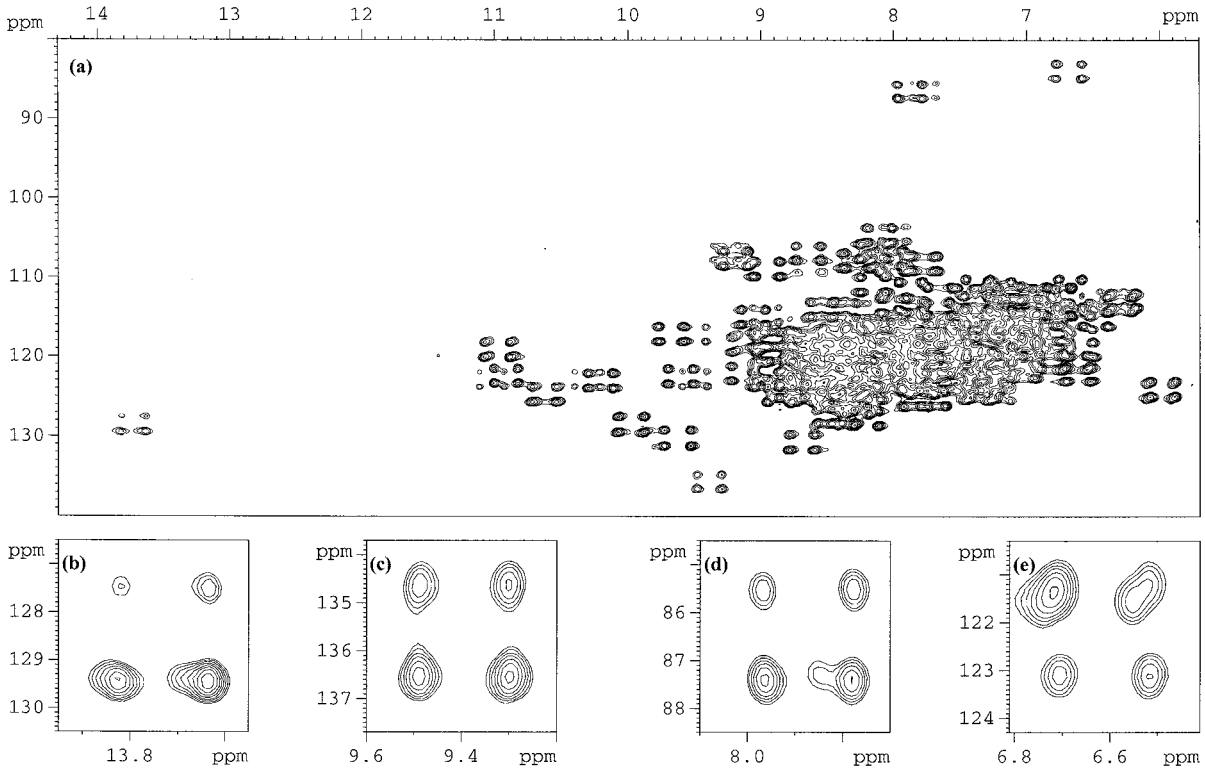

Figure 1. Experimental evidence of Paramagnetically Induced Narrowing (PIN). (a) Coupled HSQC spectrum of cyanometmyoglobin. Contour plots of four of the ^{1}H-^{15}N multiplets of the amide resonances showing differential line broadening in cyanometmyoglobin are shown with the case of intense lower right peak in the coupled HSQC (b and c), lower left (d) and upper left (e). The coupled HSQC spectrum was recorded with 256 t_1 increments with 64 scans per increment and a recycle delay of 1.8 s. The experiments were performed on a Bruker DRX-500 MHz spectrometer at pH 6.0 and 303 K on a 0.8 mM sample of cyanometmyoglobin. The protein was expressed in *Escherichia coli* from a synthetic gene encoding the amino acid sequence of sperm whale myoglobin cloned in plasmid pET21d. Apo-myoglobin was purified and reconstituted with heme dicyanide using a modified protocol adapted from published procedures. The sample preparation protocol together with the details of the heme reconstitution will be presented elsewhere.

and S spins. Relaxation terms arising due to random field terms have been omitted without loss of generality. The Redfield matrix elements in the slow molecular tumbling limit (in which $R_{1234} = R_{3412}$ are negligible) are given by

$$\begin{aligned} R_{1212,3434} &= \frac{2}{3}J_{ISIS}(0) + \frac{8}{3}\left[J_{SS}(0) + J_{eSeS}(0)\right] \\ &\quad \mp \frac{8}{3}\left[J_{ISS}(0) + J_{eSIS}(0)\right] + PRE \end{aligned} \quad (2)$$

where the spectral densities $J_{\mu\nu}(0)$ at zero frequency pertain to different interactions as follows: J_{ISIS}: IS dipolar interaction, J_{SS}: CSA relaxation of nuclear spin S, J_{eSeS}: CSR relaxation of nuclear spin S (autocorrelation term), J_{ISS}: DD×CSA cross correlated relaxation and J_{eSIS}: DD×CSR cross correlated relaxation. The term *PRE* takes care of the hyperfine contribution of the paramagnetic relaxation, namely, the dipole–dipole interaction between the nuclear and electron spins that is modulated by the electronic relaxation unlike CSR, and the Fermi contact or scalar term, represented by A, the isotropic scalar coupling constant, and is in turn given by (assuming $\omega_I, \omega_S \ll \frac{1}{T_{1e}}$) (Kowalewski, 1996):

$$\begin{aligned} PRE &= c_{fc}\left(T_{1e} + \frac{T_{1e}}{1+(\omega_e T_{1e})^2}\right) \\ &\quad + c_{dd}\left(7T_{1e} + \frac{13T_{1e}}{1+(\omega_e T_{1e})^2}\right) \\ c_{fc} &= \frac{1}{3}\left(\frac{A}{\hbar}\right)^2 S_e(S_e+1) \\ c_{dd} &= \frac{1}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \left(g_e \beta_m \gamma_S r_{eS}^{-3}\right)^2 S_e(S_e+1) \end{aligned} \quad (3)$$

The constants in the above expressions have the following meaning: T_{1e} is the longitudinal electronic relaxation time, g_e the isotropic electronic g of value

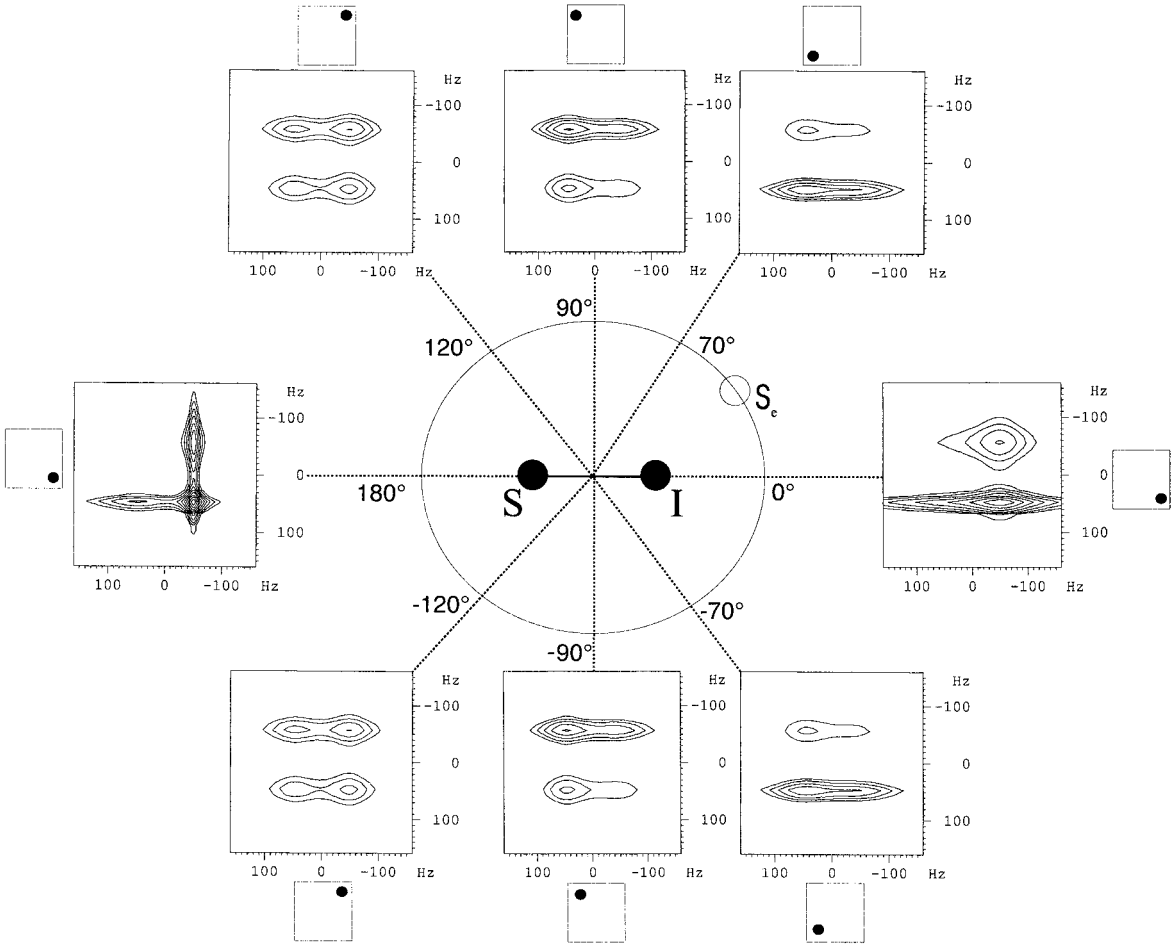

Figure 2. Simulation of the PIN effects showing contour plots of the ^{1}H-^{15}N NMR multiplets for five distinct orientations of the ^{1}H-^{15}N bond with a bond length of 1.106 Å. The parameters assumed for the simulation are as follows: A distance of 5.5 Å between the electron centre and the centre of the ^{1}H-^{15}N bond with the angles that the radial vector adapts for the various cases being indicated in the figure, a rotation correlation time of 60 ns and an electronic self relaxation time of 10^{-12} s.

2, β_m the Bohr magneton, γ_S the gyromagnetic ratio of the S spin, r_{eS} the distance between the electron and S spins, S_e the electronic spin and ω_e the Larmor frequency of the electron spin. This expression has also been arrived at by making the assumption that $T_{1e} \ll \tau_c$, τ_e with τ_c and τ_e respectively being the rotational correlation time and the exchange (chemical) time scales. The expressions for the spectral densities in the slow motion limit are given by (Wimperis and Bodenhausen, 1989; Bertini et al., 1993):

$$
\begin{aligned}
J_{ISIS}(0) &= \frac{3}{10}\left(\frac{\mu_0}{4\pi}\right)^2 \tau_c \left(\hbar \gamma_I \gamma_S r_{IS}^{-3}\right)^2 \\
J_{SS}(0) &= \frac{1}{30}\tau_c \left(\Delta\sigma_S \gamma_S B_0\right)^2 \\
J_{eSeS}(0) &= \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)^2 \\
&\quad \tau_c \left(\frac{\hbar g_e \beta_m \gamma_S \gamma_{eS} B_0 r_{eS}^{-3} S_e(S_e+1)}{kT}\right)^2
\end{aligned}
\tag{4}
$$

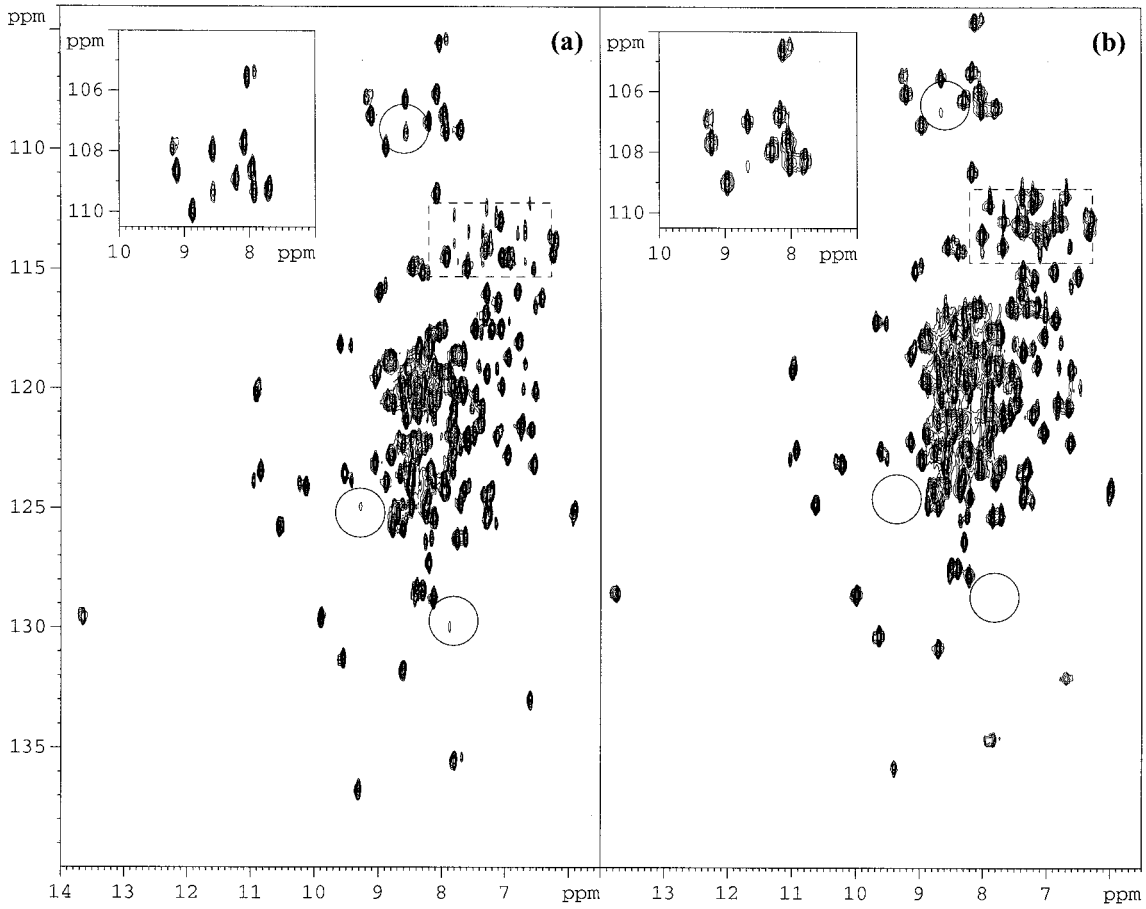

Figure 3. (a) TROSY and (b) sensitivity enhanced HSQC spectra of cyanometmyoglobin showing the appearance of additional peaks (continuous circles) in the TROSY as compared to the HSQC and the inherent resolution enhancement in the TROSY spectra as compared to the HSQC method. The dashed box highlights a region with peaks that are present in the HSQC but not in TROSY. The inset shows an expansion of one region illustrating the inherent resolution enhancement in TROSY compared to HSQC. Both TROSY and HSQC spectra were acquired under identical conditions employing 256 t_1 increments with 8 scans per increment having a recycle delay of 1.8 s. Other experimental and sample details are the same as in Figure 1.

$$J_{ISS}(0) = \frac{1}{10}\left(\frac{\mu_0}{4\pi}\right)\tau_c\hbar\Delta\sigma_S\gamma_I\gamma_S^2 B_0 r_{IS}^{-3}$$
$$\text{(assuming } \Delta\sigma_{zz} \| \vec{r}_{IS})$$

$$J_{eSIS}(0) = \frac{1}{5}\left(\frac{3\cos^2\theta_{eSIS} - 1}{2}\right)\left(\frac{\mu_0}{4\pi}\right)^2 \tau_c \left(\frac{\hbar^2\gamma_I\gamma_S^2\gamma_e g_e \beta_m B_0 r_{IS}^{-3} r_{eS}^{-3} S_e(S_e+1)}{kT}\right) \quad (4)$$

In the above, γ_e stands for the gyromagnetic ratio of the electron, k is Boltzmann's constant, T the temperature, $\Delta\sigma_s$ the CSA of spin S and θ_{eSIS} the angle between the electron-nuclear and the IS vectors. The scaling factors have been distributed between the spin and space parts such that the coefficients of CSA and CSR spectral densities in Equation 2 are the same for convenience.

Figure 2 shows contour plots of simulated ^{1}H-^{15}N cross peak multiplets, assuming different positions of the electron centre relative to the ^{1}H-^{15}N vector as indicated in the figure. This highlights the significance of CSR cross terms in determining the patterns of the four components of the ^{1}H-^{15}N cross peak. The relative amplitudes of the multiplet components clearly depend on the orientation of the ^{1}H-^{15}N vector relative to the paramagnetic centre. Hence, by comparing this simulated scenario with experimental cross peak multiplets, already a semi-quantitative knowledge can be obtained regarding the angle between the ^{1}H-^{15}N vector and the vector from the bond centre to the paramagnetic centre. A rigorous treatment of Equa-

tion 2 and quantitative comparison to experimental cross peak multiplets will allow a derivation of geometrical constraints to be used in structure refinement. Although several previous investigations have clearly laid out the principles of CSR and its cross correlation terms, few studies have been carried out exploiting them towards structure refinement (Boisbouvier et al., 1999). In this context, we believe that this relation of peak patterns and the orientation of the ^{1}H-^{15}N vector relative to the paramagnetic centre will prove fruitful. Work is in progress to carry out more thorough analysis of the line shapes, and a correlation of the numerical predictions with as yet incomplete assignments of the resonances investigated here.

In Figure 3 a sensitivity enhanced decoupled HSQC and a TROSY spectrum are compared with both experiments being performed under identical conditions. This figure, along with an inset expansion, clearly demonstrates the resolution enhancement in the TROSY spectrum. It is evident from this figure that certain peaks missing from the HSQC spectrum are present in the TROSY spectrum, indicating sensitivity enhancement for these signals. A closer inspection also reveals that some peaks appear in the HSQC spectrum but not in the TROSY spectrum. This is not surprising, since the most intense multiplet component is not always the one chosen by the usual TROSY experiment. Work is in progress to quantify the effects through a combination of four-spin-state selective experiments, each picking up one of the four possible multiplet components (Andersson et al., 1998; Weigelt, 1998). The differential narrowing will increase with higher magnetic fields and/or using high electron spin complexes. Actually sensitivity enhancement of up to 40% has been observed by Nocek et al. (2000) at 600 MHz for some amide peaks in fluoromet–hemoglobin, which is a high spin complex.

Conclusions

While progress in NMR-based assignment and structure determination of diamagnetic proteins has been substantial in the last decade, that of paramagnetic proteins has lagged behind. In view of the difficulties in structure determination of paramagnetic proteins (Bertini et al., 1993; Mäler et al., 1995; Desvaux and Gochin, 1999), we believe that this geometry dependent phenomenon can provide valuable additional geometry constraints. Cross correlation phenomena in nuclear magnetic relaxation, which have been the subject of many theoretical and experimental investigations in the history of NMR (Blicharski, 1970; Werbelow and Grant, 1977; Rance and Wright, 1986; Cuperlovic et al., 1996; Anil Kumar et al., 2000), have only recently attracted major attention in the field of biomolecular NMR (Reif et al., 1997, 2000; Yang et al., 1998; Brutscher, 2000).

Our experimental observations and theoretical calculations show that by exploiting the unique properties of cross correlated Curie spin relaxation not only sensitivity and resolution enhancement but also a new type of structural information can be obtained in paramagnetic proteins. The effect of cross correlated CSR is mostly to narrow one of the multiplet components with respect to the others and also with respect to the decoupled spectrum. We therefore choose to refer to this effect as 'paramagnetically induced narrowing' (PIN). Since it is only the additional DD$\times$CSR cross terms that are modulated by the angular disposition, the difference in the line width of the multiplet components will be only influenced by them and not by DD$\times$CSA cross terms. Assuming to a first approximation a rigid, isotropic molecular model and equal $\Delta\sigma$ for the nuclear spins, I and S, the correspondence between differential broadening and geometry is straightforward. Work is in progress to give an in-depth theoretical account of the spin dynamics involved and to develop optimised experiments and evaluation protocols to exploit this effect for NMR assignment and structure determination in paramagnetic systems.

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