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Distinguishing the Effects of Cross Correlation and *J* Coupling in COSY Spectra of Paramagnetic Proteins

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The presence of cross peaks between two protons, A and X, in a COSY spectrum is usually attributed to the presence of a J_{AX} scalar coupling. However, as discussed in detail by Wimperis and Bodenhausen (*1*), such cross peaks may also be caused by cross-correlation relaxation effects. Bertini *et al.* (*2*) proposed that the latter mechanism is largely responsible for cross peaks observed between very broad resonances in COSY spectra of paramagnetic proteins. The present Communication provides experimental evidence confirming this hypothesis and describes a simple method for distinguishing the *J*-coupling and cross-correlation mechanisms.

Because of its large magnetic moment, the electron spin is highly polarized and its Boltzmann polarization, often referred to as Curie magnetization (*3, 4*), is always parallel to the magnetic field. The magnetic moment of the Curie magnetization of the unpaired electrons of a single metal atom depends, to a good approximation, linearly on the applied static magnetic field and can be up to two orders of magnitude larger than the magnetic moment of a single proton. Hence, protons in the vicinity of the paramagnetic metal experience a strong dipolar interaction with the Curie magnetization of the unpaired electron(s), particularly for strongly paramagnetic proteins (e.g., high spin) at high magnetic fields. The spin–lattice relaxation time of unpaired electrons is frequently extremely short, reducing the otherwise dominating $J(0)$ contribution of the direct dipolar or contact interaction with the electron spin(s) for relaxing the proton. Relaxation of a proton in the vicinity of a paramagnetic metal in a protein is then primarily due to three different types of interactions: the dipolar or contact interaction between the proton and electron spin(s), the dipolar coupling between the proton and the Curie magnetization, and ^{1}H–^{1}H dipolar interactions.

Considering, for simplicity, only two protons, A and X, the local dipolar field at spin A is the vector sum of the dipolar field caused by the Curie magnetization, C, and the dipolar field of proton X. For example, if A, X, and C are arranged in a linear manner, the dipolar fields of C and X at the position of spin A add or subtract in the case where spin X is parallel ($X = |\alpha\rangle$) or antiparallel ($X = |\beta\rangle$) to the static magnetic field. Consequently, spin A will experience a stronger local field when spin X is in the $|\alpha\rangle$ spin state compared to $X = |\beta\rangle$, and therefore it will relax faster. The differences in T_2 for the general nonlinear case are given by Bertini *et al.* (*2*).

In-phase transverse A-spin magnetization immediately after the initial 90°_x pulse of the COSY experiment is described by the sum of the two doublet components

$$\mathbf{A}_y = (\mathbf{A}_y + 2\mathbf{A}_y\mathbf{X}_z)/2 + (\mathbf{A}_y - 2\mathbf{A}_y\mathbf{X}_z)/2, \qquad [1]$$

where the first and second terms on the right-hand side of Eq. [1] correspond to the doublet components with $X = |\alpha\rangle$ and $X = |\beta\rangle$, respectively, and relax with transverse relaxation times $T_{2A}^{X=\alpha}$ and $T_{2A}^{X=\beta}$. These components precess at different frequencies if $J_{AX} \neq 0$, although, as will be shown below, this is not an essential prerequisite for the generation of COSY cross peaks.

Assuming, temporarily, that spin A is on resonance, the effect of transverse relaxation during t_1 is described by

$$\mathbf{A}_y \xrightarrow{t_1} (\mathbf{A}_y + 2\mathbf{A}_y\mathbf{X}_z)/2 \exp(-t_1/T_{2A}^{X=\alpha}) + (\mathbf{A}_y - 2\mathbf{A}_y\mathbf{X}_z)/2 \exp(-t_1/T_{2A}^{X=\beta}) \qquad [2a]$$

or

$$\mathbf{A}_y \xrightarrow{t_1} \mathbf{A}_y[\exp(-t_1/T_{2A}^{X=\alpha}) + \exp(-t_1/T_{2A}^{X=\beta})]/2 + \mathbf{A}_y\mathbf{X}_z[\exp(-t_1/T_{2A}^{X=\alpha}) - \exp(-t_1/T_{2A}^{X=\beta})]. \qquad [2b]$$

The $\mathbf{A}_y\mathbf{X}_z$ term on the right-hand side of Eq. [2b] denotes the antiphase A-spin magnetization which is transferred into antiphase X-spin magnetization by the subsequent 90°_x pulse. Assuming that $(T_{2A}^{X=\alpha} - T_{2A}^{X=\beta})/T_{2A} \ll 1$, where $1/T_{2A} = (1/T_{2A}^{X=\alpha} + 1/T_{2A}^{X=\beta})/2$, the $\mathbf{A}_y\mathbf{X}_z$ term reaches a maximum value when $t_1 \approx T_{2A}$. If, in addition, $t_1 \ll 1/J_{AX}$, the effect of J_{AX} coupling on t_1 evolution may simply be included in Eq. [2b] according to

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$$\mathbf{A}_y \xrightarrow{t_1} \mathbf{A}_y \exp(-t_1/T_{2\mathrm{A}})\cos(\pi J_{\mathrm{AX}} t_1) - 2\mathbf{A}_x\mathbf{X}_z \sin(\pi J_{\mathrm{AX}} t_1) + 2\mathbf{A}_y\mathbf{X}_z f_{\mathrm{A}}(t_1), \quad [3a]$$

where

$$\begin{aligned} f_{\mathrm{A}}(t_1) &= [\exp(-t_1/T_{2\mathrm{A}}^{\mathrm{X}=\alpha}) - \exp(-t_1/T_{2\mathrm{A}}^{\mathrm{X}=\beta})]/2 \\ &= \exp(-t_1/T_{2\mathrm{A}})\sinh(\pi\delta_{\mathrm{A}} t_1) \\ &\approx \exp(-t_1/T_{2\mathrm{A}})\sin(\pi\delta_{\mathrm{A}} t_1) \quad [3b] \end{aligned}$$

and

$$\delta_{\mathrm{A}} = (T_{2\mathrm{A}}^{\mathrm{X}=\alpha} - T_{2\mathrm{A}}^{\mathrm{X}=\beta})/2\pi(T_{2\mathrm{A}}^{\mathrm{X}=\alpha} T_{2\mathrm{A}}^{\mathrm{X}=\beta}). \quad [3c]$$

The terms $2\mathbf{A}_x\mathbf{X}_z$ and $2\mathbf{A}_y\mathbf{X}_z$ in Eq. [3a] result in the *J*-correlation and cross-correlation cross-peak components in the COSY spectrum, respectively. The ratio of the *J*- and cross-correlation contributions to the COSY cross-peak intensity depends on the ratio J/δ. Note that the cross-correlation component is in phase with respect to the diagonal component ($\mathbf{A}_y$), whereas the *J*-correlation component ($2\mathbf{A}_x\mathbf{X}_z$) is out of phase by 90° (*5*). This difference forms the basis for distinguishing the two contributions.

Conducting two experiments for each t_1 value, with phases *x* and *y* for the first 90° pulse and phase *y* for the second 90° proton pulse, and acquiring the data in the well-known hypercomplex manner (*6, 7*) give the signal

$$\begin{aligned} S(t_1, t_2) = M_0\{&\cos(\pi J_{\mathrm{AX}} t_1)\cos(\pi J_{\mathrm{AX}} t_2)\exp(j\Omega_{\mathrm{A}} t_1) \\ &\times \exp(i\Omega_{\mathrm{X}} t_2)\exp[-(t_1/T_{2\mathrm{A}} + t_2/T_{2\mathrm{X}})] + \sin(\pi J_{\mathrm{AX}} t_1) \\ &\times \sin(\pi J_{\mathrm{AX}} t_2)\exp(j\Omega_{\mathrm{A}} t_1)\exp(i\Omega_{\mathrm{X}} t_2) \\ &\times \exp[-(t_1/T_{2\mathrm{A}} + t_2/T_{2\mathrm{X}})] + f_A(t_1)\sin(\pi J_{\mathrm{AX}} t_2) \\ &\times \exp[j(\Omega_{\mathrm{A}} t_1 + \pi/2)]\exp(i\Omega_{\mathrm{X}} t_2)\exp(-t_2/T_{2\mathrm{X}}) \\ &+ \sin(\pi J_{\mathrm{AX}} t_1) f_{\mathrm{X}}(t_2)\exp[j\Omega_{\mathrm{A}} t_1]\exp[i(\Omega_{\mathrm{X}} t_2 + \pi/2)] \\ &\times \exp(-t_1/T_{2\mathrm{A}}) + f_{\mathrm{A}}(t_1) f_{\mathrm{X}}(t_2)\exp[j(\Omega_{\mathrm{A}} t_1 + \pi/2)] \\ &\times \exp[i(\Omega_{\mathrm{X}} t_2 + \pi/2)]\}, \quad [4] \end{aligned}$$

where *i* and *j* denote the imaginary components in the t_2 and t_1 dimensions, respectively, $f_{\mathrm{X}}(t_2) = [\exp(-t_2/T_{2\mathrm{X}}^{\mathrm{A}=\alpha}) - \exp(-t_2/T_{2\mathrm{X}}^{\mathrm{A}=\beta})]/2$, and $1/T_{2\mathrm{X}} = (1/T_{2\mathrm{X}}^{\mathrm{A}=\alpha} + 1/T_{2\mathrm{X}}^{\mathrm{A}=\beta})/2$. Thus, a fraction of magnetization $2\mathbf{A}_z\mathbf{X}_x$ after the COSY mixing pulse results in a fraction $f_{\mathrm{X}}(t_2)$ of in-phase $\mathbf{X}_x$ magnetization.

Using approximations analogous to the one mentioned above for $f_{\mathrm{A}}(t_1)$, $f_{\mathrm{X}}(t_2)$ may be written as

$$f_{\mathrm{X}}(t_2) \approx \exp(-t_2/T_{2\mathrm{X}})\sin(\pi\delta_{\mathrm{X}} t_2), \quad [5a]$$

with

$$\delta_{\mathrm{X}} = (T_{2\mathrm{X}}^{\mathrm{A}=\alpha} - T_{2\mathrm{X}}^{\mathrm{A}=\beta})/2\pi(T_{2\mathrm{X}}^{\mathrm{A}=\alpha} T_{2\mathrm{X}}^{\mathrm{A}=\beta}). \quad [5b]$$

Using the identity $\sin(\alpha) = [\exp(i\alpha) - \exp(-i\alpha)]/2i$, Eq. [4] may be rewritten as

$$S(t_1, t_2) = (1/4)M_0\exp[-(t_1/T_{2\mathrm{A}} + t_2/T_{2\mathrm{X}})] \times (P + Q + R + S + T) \quad [6]$$

with

$$\begin{aligned} P = \{&\exp[j(\Omega_{\mathrm{A}} + \pi J_{\mathrm{AX}})t_1] \\ &+ \exp[j(\Omega_{\mathrm{A}} - \pi J_{\mathrm{AX}})t_1]\}\{\exp[i(\Omega_{\mathrm{X}} + \pi J_{\mathrm{AX}})t_2] \\ &+ \exp[i(\Omega_{\mathrm{X}} - \pi J_{\mathrm{AX}})t_2]\} \\ Q = \{&\exp[j(\Omega_{\mathrm{A}} + \pi J_{\mathrm{AX}})t_1 - j\pi/2] \\ &- \exp[j(\Omega_{\mathrm{A}} - \pi J_{\mathrm{AX}})t_1 - j\pi/2]\} \\ &\times \{\exp[i(\Omega_{\mathrm{X}} + \pi J_{\mathrm{AX}})t_2 - i\pi/2] \\ &- \exp[i(\Omega_{\mathrm{X}} - \pi J_{\mathrm{AX}})t_2 - i\pi/2]\} \\ R = \{&\exp[j(\Omega_{\mathrm{A}} + \pi\delta_{\mathrm{X}})t_1] \\ &- \exp[j(\Omega_{\mathrm{A}} - \pi\delta_{\mathrm{X}})t_1]\}\{\exp[i(\Omega_{\mathrm{X}} + \pi J_{\mathrm{AX}})t_2 - i\pi/2] \\ &- \exp[i(\Omega_{\mathrm{X}} - \pi J_{\mathrm{AX}})t_2 - i\pi/2]\} \\ S = \{&\exp[j(\Omega_{\mathrm{A}} + \pi J_{\mathrm{AX}})t_1 - j\pi/2] \\ &- \exp[j(\Omega_{\mathrm{A}} - \pi J_{\mathrm{AX}})t_1 - j\pi/2]\}\{\exp[i(\Omega_{\mathrm{X}} + \pi\delta_{\mathrm{A}})t_2] \\ &- \exp[i(\Omega_{\mathrm{X}} - \pi\delta_{\mathrm{A}})t_2]\} \\ T = \{&\exp[j(\Omega_{\mathrm{A}} + \pi\delta_{\mathrm{X}})t_1] \\ &- \exp[j(\Omega_{\mathrm{A}} - \pi\delta_{\mathrm{X}})t_1]\}\{\exp[i(\Omega_{\mathrm{X}} + \pi\delta_{\mathrm{A}})t_2] \\ &- \exp[i(\Omega_{\mathrm{X}} - \pi\delta_{\mathrm{A}})t_2]\}. \end{aligned}$$

Terms *P* and *Q* in Eq. [6] represent the A-spin diagonal peak and the *J* contribution to the A → X cross peak, respectively. The *J*-coupling contribution (term *Q*) gives rise to the well-known antiphase cross-peak pattern which is $\pi/2$ out of phase in both the F_1 and the F_2 dimensions with respect to the in-phase diagonal multiplet. Term *T* is due exclusively to cross correlation and also gives rise to an antiphase cross-peak pattern which is, however, *in phase* with respect to the diagonal multiplet. Note that the intensity of the cross-correlation antiphase multiplet equals the intensity of a *J*-coupling multiplet with an unresolved active *J* coupling δ_{X} in the F_1 dimension and δ_{A} in the F_2 dimension. Interestingly, δ_{A} and δ_{X} are generally different and can even be of opposite signs. As can be seen from Eq. [6], if δ_{A} and δ_{X} are of the same sign, the two cross-multiplet components on a line parallel to the diagonal of the COSY spectrum have the same phase as the diagonal resonances, i.e., positive. If δ_A and δ_X are of opposite sign, these two cross-peak multiplet components are negative and the other two are positive.

The mixed terms R and S result from antiphase magnetization buildup during t_1 caused by cross correlation (term R) and J dephasing (term S) and from the rephasing after the COSY 90°_y mixing pulse due to J coupling (term R) and by cross correlation (term S). To a good approximation, the R, S, and T terms relate to one another as

$$R \approx (J_{AX}/\delta_A)\exp(-i\pi/2)T \qquad [7a]$$

$$S \approx (J_{AX}/\delta_X)\exp(-j\pi/2)T. \qquad [7b]$$

The R term is 90° out of phase in the F_2 dimension compared to the doubly absorptive T term, and the S term is 90° out of phase in the F_1 dimension. In principle, the relative magnitude and sign of J_{AX}, δ_A, and δ_X therefore follow from the additional phase corrections needed to phase the cross multiplet to pure absorption, starting from a spectrum with a purely absorptive diagonal. In practice, for cross peaks with a low signal-to-noise ratio, the additional phase corrections cannot be measured precisely, prohibiting accurate measurement of the R/T and S/T ratios.

At first sight it might appear counterintuitive that cross correlation in the absence of J correlation can give rise to a COSY cross peak with four multiplet components. As pointed out by Wimperis and Bodenhausen, a cross-correlation cross peak between spins A and X results from the difference between broad and narrow components of the A- and X-spin lineshapes, which should be 90° out of phase relative to the diagonal resonance. As illustrated in Fig. 1, this analysis is in full agreement with the results presented here. Figure 1C shows the difference between two absorptive lines with different linewidths but the same integrated intensity, centered at the same chemical shift. After the 90° phase correction is applied to this difference spectrum (Fig. 1D) it has the same appearance as the sum of two absorptive antiphase resonances of identical shape, but with a frequency difference that is small compared to their linewidth (Fig. 1G). This pictorial illustration is equivalent to substituting $\sin(\pi\delta_A t_1) = [\exp(i\pi\delta_A t_1) - \exp(-i\pi\delta_A t_1)]/2$ into Eq. [3b]. So, cross correlation results in a cross-peak multiplet pattern that resembles an absorptive COSY J cross peak. In contrast, cross peaks caused by J coupling are dispersive when the diagonal is phased to pure absorption in a regular (i.e., not double-quantum-filtered) COSY spectrum.

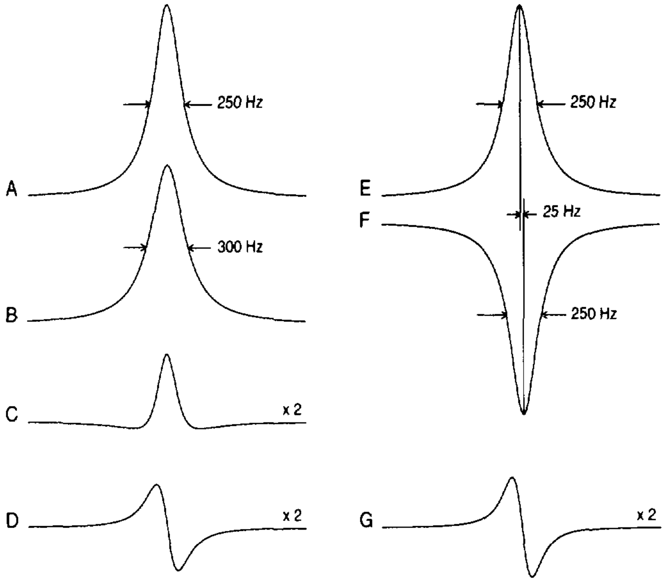

FIG. 1. Lineshape simulations for the case of cross correlation (A–D) and J coupling (E–G). (A) and (B) are 250 and 300 Hz-wide Lorentzian lines of identical intensity, (C) is their difference, and (D) is the result after 90° phase correction. (E) and (F) are two 250 Hz-wide antiphase Lorentzians, displaced by 25 Hz, and (G) is their sum.

The approach to separate the J- and cross-correlation contributions is demonstrated for the hyperfine-shifted resonances in resting-state horseradish peroxidase (HRP), a protein for which cross peaks in the absolute-value-mode COSY spectrum have been observed and identified previously (*8*). A phase-sensitive COSY spectrum was recorded at 55°C on a Bruker AMX-360 spectrometer, using a sample concentration of 3 mM in D_2O, pH 7. The spectrum was recorded with the sequence $90^\circ_{\phi 1}$—t_1—$90^\circ_{\phi 2}$—Acquire (ψ), with $\phi_1 = x, -x, y, -y, -x, x, -y, y$, $\phi_2 = y, y, -x, -x, -y, -y, x, x$, and $\psi = y, -y, -x, x, -y, y, x, -x$. Quadrature in the t_1 dimension was obtained by incrementing ϕ_1 in the regular State–TPPI manner (*7*). The spectrum results from a $128^* \times 256^*$ data matrix, where N^* denotes N complex data points. Acquisition times were 3.6 ms in both dimensions, with 32,000 scans per complex t_1 increment, a delay time of 20 ms between scans (including t_2 data acquisition), and a total measuring time of 25 h. Data were apodized by a 50°-shifted squared sine-bell filter and zero filled to yield a 512 × 1024 data matrix for the absorptive region of the spectrum, with a digital resolution of 65 Hz.

The 50–30 ppm region of the phase-sensitive COSY spectrum of HRP is shown in Fig. 2. The chemical shifts of the two geminal protons 7-Hα and 7-Hα' are sufficiently different to allow unambiguous observation of the cross peak between them, despite a low cross-peak to diagonal-peak intensity ratio of about 50:1. The cross peaks show an up–down pattern resembling an absorptive AX COSY cross multiplet when the diagonal is phased to pure absorption. This indicates that the T term dominates in Eq. [6], i.e., that the cross peaks are primarily due to cross correlation, as predicted by Bertini *et al.* (*2*). However, some asymmetry in the cross peak is also visible and is caused by the presence of nonnegligible R- and S-term contributions.

From the X-ray crystal structure of cytochrome c peroxidase (*9*), the distances between 7-Hα (spin A) and the Fe

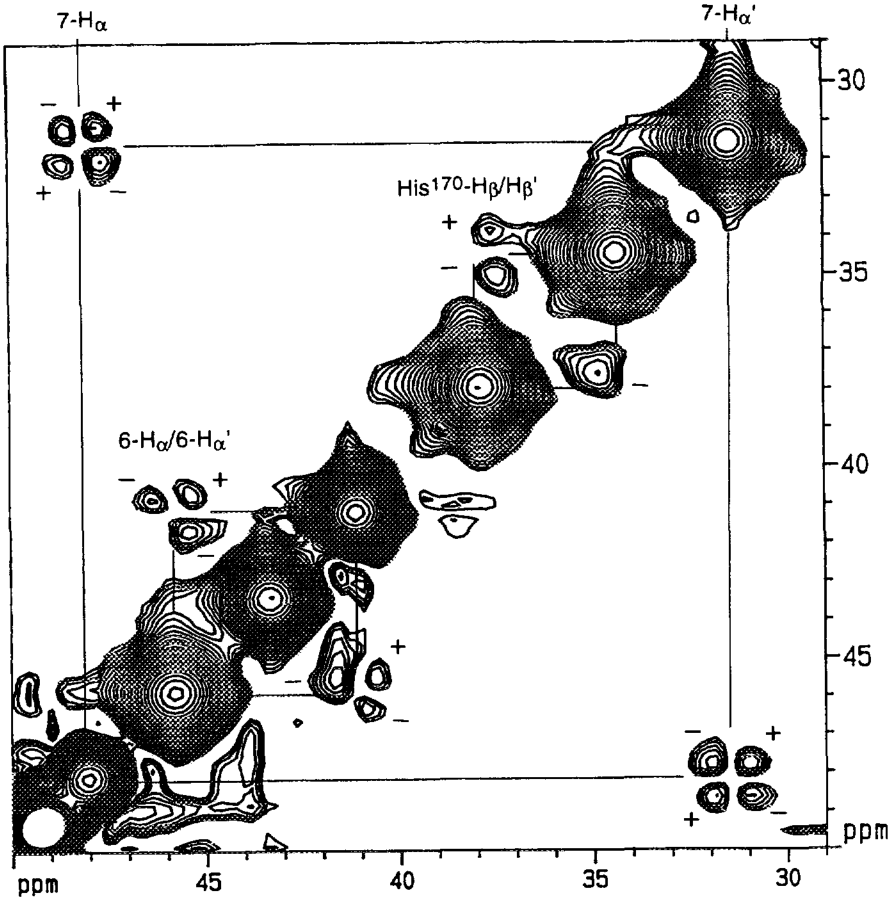

FIG. 2. Downfield region of the 360 MHz phase-sensitive COSY spectrum of horseradish peroxidase in D_2O, at pH 7.0 and 55°C. The nearly absorptive antiphase cross peak between the well-resolved 7-Hα and 7-Hα′ resonances shows the predominance of the cross-correlation effect. The slight asymmetry of the cross peak is due to nonnegligible *J*-coupling effects. The diagonal is phased to be positive, and positive and negative components of the cross-peak multiplet are respectively marked "+" and "−". Cross peaks between $\text{His}^{170}\text{-C}^{\beta}\text{H}$ and $\text{-C}^{\beta}\text{H}'$ and between 6-Hα and 6-Hα′ of the heme are distorted due to their proximity to the ~50 times more intense diagonal resonances.

nucleus (C) and between 7Hα′ (X) and C are 6.37 and 5.84 Å, respectively, with an AX distance of 1.77 Å and θ_{AXC} and θ_{XAC} angles of 99° and 65°. Assuming a 15 ns rotational correlation time, the $J(0)$ AX dipolar contribution to the transverse relaxation rate, $R_{DD(AX)}$, is calculated to be 128 s^{-1}. The Curie relaxation mechanism contributes a fraction $R_{CR(A)} = 453\ s^{-1}$ to the transverse relaxation rate of spin A, and $R_{CR(X)} = 760\ s^{-1}$ for spin X. The cross-correlation contribution, R_{CC}, to the transverse relaxation rate equals $-201\ s^{-1}$ for spin A and $-132\ s^{-1}$ for spin X. The T_1 values of A and X are dominated by $J(\omega)$ and $J(2\omega)$ terms caused by the direct dipolar and contact interaction with the very rapidly relaxing electron spins. T_1 values measured at 360 MHz for 7-Hα and 7-Hα′ are 5 and 4.2 ms, respectively, increasing the transverse relaxation rates of spins A and X by 200 and 240 s^{-1}, respectively. Based on these numbers, the transverse relaxation times are then expected to be $T_{2A}^{X=\alpha} = 1.7$ ms, $T_{2A}^{X=\beta} = 1.0$ ms, $T_{2X}^{A=\alpha} = 1.0$ ms, and $T_{2X}^{A=\beta} = 0.8$ ms. These calculated T_2 values are in reasonable agreement with the average T_2 values measured for these two spins ($T_{2A} = 1.1 \pm 0.1$ ms, $T_{2X} = 1.0 \pm 0.1$ ms). The "apparent *J* couplings," δ_A and δ_X (cf. Eqs. [3c] and [5b]), are then equal to 65 and 40 Hz, respectively, which is considerably larger than the geminal J_{AX} coupling of ~14 Hz. As seen from Eq. [7] and these numbers, the cross-correlation contribution (term T) is approximately 13 times stronger than the *J*-coupling contribution (term Q), but the mixed terms, R and S, are only about 3 to 4 times weaker than T and cause the observed cross peak to deviate slightly from a purely absorptive and symmetric up–down multiplet pattern.

We have shown that the effects of cross correlation and *J* coupling in COSY spectra of paramagnetic proteins can readily be distinguished by considering the phase of the cross peak relative to that of the diagonal peak. The relative sign of the cross-correlation contributions to the two spins can also be extracted from the cross-peak shape: If the two cross-peak components on a line parallel to the diagonal have the same sign as the diagonal resonances, the cross-correlation contributions are of the same sign. If the two multiplet components on a line perpendicular to the diagonal have the same sign as the diagonal resonances, the cross-correlation contributions to the relaxation of the two spins are opposite.

The procedure described above has important consequences for quantitative NMR studies of a range of important high-spin metalloproteins which exhibit strong Curie relaxation, including resting-state heme peroxidase (*8*), as well as ferrous or deoxymyoglobins and hemoglobins (*10*), ferricytochromes c′ (*11, 12*), Co(II)-substituted Zn enzymes (*13*), and trivalent lanthanide-substituted calcium-binding proteins (*14*) for which 2D NMR studies have been reported or are in progress. First, it will be possible to establish the critical scalar connectivities needed to identify spin systems during the sequence-specific assignment process (*15*). The scalar connectivity is even more crucial in a paramagnetic than diamagnetic system since the usual connection between chemical shift and functional group is lost. Second, the contribution from cross correlation could yield valuable data on the geometry of the molecular fragment.

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