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Strong Coupling Effects in the Homonuclear RELAY Experiment, with Applications to Leucine Spin Systems of Octanoyl-Acyl Carrier Protein

LEWIS E. KAY,* PAUL-JAMES JONES,† AND J. H. PRESTEGARD†

*Department of Molecular Biophysics and Biochemistry and †Department of Chemistry, Yale University, New Haven, Connecticut 06511

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The wide variety of 2D NMR experiments used today in the assignment of spectra of complex molecules has been designed and interpreted largely on the basis of coherence transfer selection rules developed first by Ernst and co-workers (*1, 2*). The rules were derived, however, for weakly coupled, nonrelaxing spin systems, and violations of these rules have now been shown to arise in cases where such conditions do not prevail. For example, strong scalar coupling gives rise to intensity anomalies and additional resonances in *J*-resolved (*3*), NOESY (*4*), and multiple-quantum-filtered COSY (*5, 6*) spectra. It has also been shown that differential relaxation of transitions in degenerate spin systems (methyl groups, for example) gives rise to "forbidden" peaks in multiple-quantum (*5, 7–9*) and multiple-quantum-filtered COSY spectra (*5, 7*). Understanding the origin of such peaks is valuable both because it can avoid possible misassignments resulting from a first-order interpretation and because it can provide additional useful information.

In a recent communication, Otter *et al.* (*10*) reported on the appearance of unexpected cross peaks in homonuclear RELAY experiments on a small peptide. This report is of great interest to us because of the utility RELAY experiments have shown in spectral assignments on a protein under study in our laboratory, acyl carrier protein (ACP, 8850 daltons) (*11, 12*). Figure 1 shows a contour plot of the cross peaks from several leucines in an acylated form of ACP, octanoyl-ACP. Of note are the cross peaks between the δCH_3's and the αCH of leucine 32 as well as leucine 42 which are not expected on the basis of a one-step relay transfer (*13*). We would like to present here a theoretical interpretation of the origin of these peaks and an illustration of their utility in spectral assignment.

There are several potential sources of these unexpected peaks. For example, an imperfect refocusing pulse applied during the mixing time could cause transfer of magnetization between the δCH_3's and αCH of leucine via a triple relay. However, a rigorous product-operator calculation (*14, 15*) of this case predicts chemical-shift dependent intensities of forbidden peaks for Leu 32 and 37 which do not agree with the intensities observed experimentally. In addition it is highly unlikely that nonuniform T_2 relaxation (*5, 7–9*) could lead to the appearance of such forbidden peaks. If such were the case, the ratio of the forbidden peak intensity to the relay peak intensity

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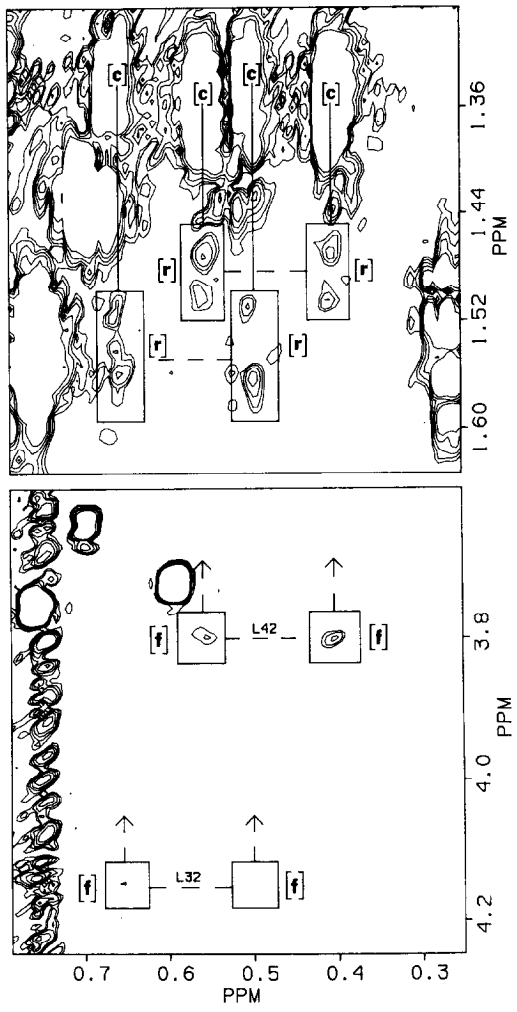

FIG. 1. Portions of the relayed-COSY spectrum illustrating the γCH–$\delta(CH_3)_2$ COSY-type cross peaks (c), the βCH_2–$\delta(CH_3)_2$ RELAY cross peaks (r), and the αCH–$\delta(CH_3)_2$ forbidden cross peaks (f) for two leucine residues from octanoyl-ACP. The spectrum was acquired from a 13 m*M* sample in 35 m*M* KH_2PO_4 buffer, pH 6.35, with a mixing time of 50 ms. Eight hundred t_1 points were acquired, 352 scans per file, with a recycle time of 1.1 s. The spectrum was processed in a magnitude format using an unshifted sine-bell function extending over 1K points in t_2, and an unshifted skewed sine-bell with a skew of 0.3 extending over 800 points in t_1.

would be expected to increase on going from a small peptide to a macromolecule because multiexponential relaxation effects increase as a function of increasing molecular correlation time (*5, 9*). A comparison of the data of Otter *et al.* (*10*) with our data shows no increase in the ratio of forbidden peak intensity to allowed relay peak intensity.

We propose that the origin of such peaks is due to strong coupling between the β and γ protons of leucine. This explanation is consistent with the experimental obser-

vation that for Leu 32, Leu 37, and Leu 42 with $J/(\nu_\beta - \nu_\gamma)$ values of 0.04, 0.2, and 0.4, respectively, the intensities of the forbidden peaks increase, in the ratio 1:5:10. Moreover, we note that a recent interpretation of an unexpected relay cross peak between the protons at the ϵ_3 and ζ_2 positions of a tryptophan in terms of strong coupling effects supports this explanation (*16*).

One can gain theoretical insight into the origin of these unexpected cross peaks by considering an AMNX spin system with spins M and N strongly coupled and tracing the path that the magnetization takes on going from spin A to spin X in a RELAY experiment. A product operator treatment (*14*) which includes the effects of strong coupling shows that two possible pathways for the transfer of magnetization from spin A to spin X are

$$A_0 \xrightarrow{90^\circ} A_{-1} \xrightarrow{t_1} 2A_{-1}M_0 \xrightarrow{90^\circ} 2A_0M_{\pm 1} \xrightarrow{\tau-180^\circ-\tau} 2N_{\pm 1}X_0 \xrightarrow{90^\circ} 2N_0X_{+1} \xrightarrow{t_2} [1]$$

and

$$A_0 \xrightarrow{90^\circ} A_{-1} \xrightarrow{t_1} 2A_{-1}N_0 \xrightarrow{90^\circ} 2A_0N_{\pm 1} \xrightarrow{\tau-180^\circ-\tau} 2N_{\pm 1}X_0 \xrightarrow{90^\circ} 2N_0X_{+1} \xrightarrow{t_2} [2]$$

In Eqs. [1] and [2] we have assumed N-type selection and

$$A_{-1} = -(A_x + iA_y)/\sqrt{2}$$
$$A_0 = A_z$$
$$A_{+1} = (A_x - iA_y)/\sqrt{2}. \qquad [3]$$

Thus the term $2A_{-1}M_0$ represents A magnetization of coherence order -1 out of phase with respect to M magnetization (*14, 15*).

We have also simulated RELAY experiments for an AMNX spin system with chemical shifts and coupling constants similar to those found in leucine spin systems. This is done as a function of $J_{MN}/(\nu_M - \nu_N)$, for fixed J_{MN}, using a computer program developed in our laboratory and discussed in a previous publication (*4*). Figure 2 shows the results of this simulation. The intensity of a forbidden peak appearing at the shift of A in f_1 and X in f_2 reaches a maximum at scalar coupling to chemical-shift ratios of 0.4 and 0.3 for mixing times of 50 and 40 ms, respectively, and falls to zero as the spectrum approaches the first-order limit. In addition, the simulations show that the transfer of magnetization from spin A to X becomes less efficient as $J_{MN}/(\nu_M - \nu_N)$ ratios increase from approximately 0.4 to 0.8. Although the intensity of the forbidden peaks in this simulation is at most 7% of the intensity of the diagonal peaks, it is quite conceivable that for the actual leucine spin system with six δ-methyl protons capable of transferring magnetization, the forbidden peaks could be substantially larger. This is observed in the experimental data where the forbidden peaks can be as much as 30% as intense as the allowed relay peaks. In addition, the simulations indicate that the intensities of these unexpected cross peaks are exquisitely sensitive

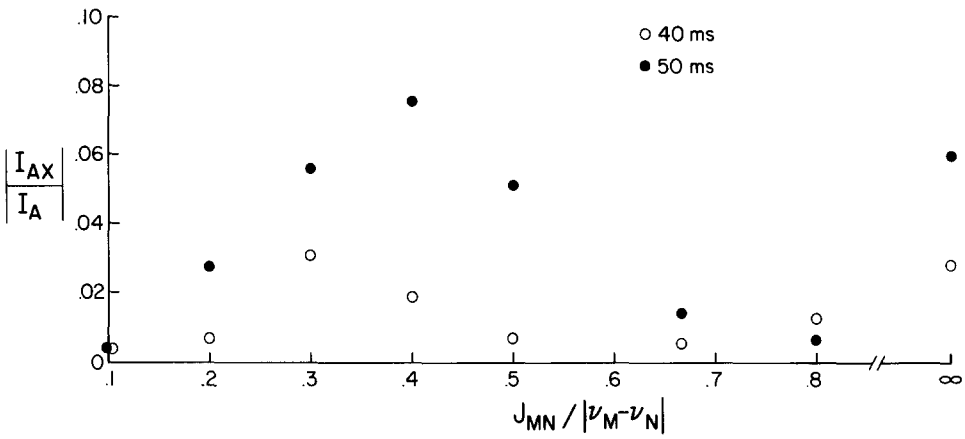

FIG. 2. Intensity of the forbidden A–X relay peak normalized to the diagonal A peak as a function of $J_{MN}/(\nu_M - \nu_N)$ for an AMNX spin system. Mixing times of 40 and 50 ms were used. δ_A = 725 Hz, δ_N = 375 Hz, δ_X = 75 Hz, J_{AM} = 5.0 Hz, J_{MN} = 10.0 Hz, J_{NX} = 6.0 Hz. δ_M was varied to give the $J_{MN}/(\nu_M - \nu_N)$ ratios indicated in the figure. The pulse sequence and associated phase cycling is indicated in Ref. (*13*).

to the mixing time chosen which is in agreement with the observations of Otter *et al.* (*10*).

When they are expected and understood, the appearance of forbidden peaks in RELAY spectra can be useful in establishing connectivities between remote parts of a spin system. The spin system of leucine is a case in point. Although the NH–Hα–Hβ fragments of leucine are usually readily assigned through the use of traditional sequential assignment strategies, extension of these assignments to peripheral side-chain resonances usually proves difficult. The assignment problem arises in attempting to connect the γCH–$\delta(CH_3)_2$ fragment, which is assigned on the basis of COSY or RELAY data (*17*), to the corresponding NH–Hα–Hβ fragment. Several factors contribute to this difficulty. First, since the βCH_2 and γCH proton resonances are often nearly degenerate, the cross peaks connecting these resonances in a COSY experiment are near the diagonal and partially obscured. Second, even if the βCH_2 and γCH resonances are well separated, the cross peaks connecting these resonances are of very low intensity due to the high degree of multiplicity associated with the γCH resonance. Normal RELAY data often does not circumvent these problems either. For example, due to the near degeneracy of the βCH_2 and γCH resonances, the βCH_2–$\delta(CH_3)_2$ cross peaks are often obscured by the intense γCH–$\delta(CH_3)_2$ COSY-type peaks, while, αCH–γCH cross peaks are of very low intensity due to the large multiplicity of the γCH resonance. Recent schemes for connecting remote parts of spin systems which involve coherence transfer of magnetization via isotropic mixing are very encouraging (*18*). However, the pulse sequences involved are complex and it may prove difficult to effectively transfer magnetization between spins that are far apart in chemical shift without using prohibitively large RF irradiation power levels and long mixing times.

The presence of unexpected cross peaks between the αCH–$\delta(CH_3)_2$ resonances in a conventional RELAY experiment allows a direct connection between the two known

halves of the leucine spin system. In the case of Leu 32 and Leu 42 of octanoyl-ACP, weak Hβ–$\delta(CH_3)_2$ cross peaks are observed in the RELAY experiment and hence the entire spin systems of these residues could be assigned independently of the appearance of the forbidden peaks. However, the presence of forbidden RELAY peaks has allowed the complete assignment of Leu 37 for which Hβ–$\delta(CH_3)_2$ cross peaks are not observed.

Finally we wish to emphasize that the complete assignment of hydrophobic amino acid side chains, in particular, may prove crucial for a successful protein structure determination by NMR. Since, in general, it is the hydrophobic amino acids which occur in the interior of proteins where the probability of contact between nonsequential amino acids is the greatest, NOEs to the side chains of these residues will provide potentially valuable distance constraints.

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