Two-Dimensional ^1H NMR Studies of Histidine-Containing Protein from *Escherichia coli*. 2. Leucine Resonance Assignments by Long-Range Coherence Transfer†

Rachel E. Klevit‡ and Gary P. Drobny*

Department of Chemistry, University of Washington, Seattle, Washington 98195

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ABSTRACT: Sequence-specific assignments of the NH, $C^{\alpha}H$, and $C^{\beta}H$ resonances in the NMR spectrum of the histidine-containing protein (HPr) from *Escherichia coli* are complete [Klevit, R. E., Drobny, G. P., & Waygood, E. B. (1986) *Biochemistry* (first paper of three in this issue)]. In addition, the $C^{\gamma}H_3$ resonances of valyl, threonyl, and isoleucyl residues have been assigned by two-dimensional relayed coherence transfer (RELAY) experiments. In order to rigorously assign the resonances from longer side chains such as leucines, long-range transfer experiments have been applied to HPr. Coherence transfers via isotropic mixing within large spin systems were accomplished by multiple pulse trains applied during the mixing time of a two-dimensional experiment.

In the preceding paper, we presented sequence-specific resonance assignments for an 85-residue protein, HPr[1] from *Escherichia coli*. The strategy used three types of homonuclear two-dimensional NMR experiments, COSY, NOESY, and RELAY, and resulted in the assignments of NH, $C^{\alpha}H$, $C^{\beta}H$, and some $C^{\gamma}H$ resonances. On the basis of these assignments, both the secondary structure and the overall tertiary fold of the protein can be determined (Klevit & Waygood, 1986). Recent results of algorithms designed to obtain protein structures from NOE intensities (Kaptein et al., 1985; Havel & Wüthrich, 1985; Braun & Gō, 1986) show that it is indeed possible to obtain refined structures from NMR results. These procedures require the identification of as many NOESY cross peaks as possible. In many instances, however, cross peaks cannot be identified due to the lack of assignments of side-chain resonances, and useful structural information therefore remains untapped. The tertiary fold of a protein is, for the most part, defined by long-range NOEs (i.e., between protons that are far from each other in the primary sequence) that frequently involve protons at the ends of side chains.

In the process of analyzing NOESY spectra of HPr in on-going structural studies of the protein, we noticed that the $C^{\delta}H_3$ resonances of leucine side chains gave many NOE cross peaks. However, it is virtually impossible to rigorously connect the $C^{\delta}H_3$ and $C^{\delta'}H_3$ resonances to the sequentially assigned leucine $C^{\beta}H$ resonances in a COSY or RELAY experiment because the $C^{\beta}H$–$C^{\gamma}H$ cross peaks occur in a poorly resolved spectral region near the diagonal. In general, one or the other leucyl methyl resonances will give an intraresidue NOE to either a $C^{\beta}H$ or $C^{\alpha}H$ resonance, but the assignment of leucyl methyls on the basis of such an NOE may be problematic for leucines in closely packed hydrophobic cores where other leucine side chains may be found in close proximity. Although in principle the RELAY experiment can be extended in order to observe two-step relayed coherence transfer (Eich et al., 1982), this technique does not appear to be generally useful for proteins, presumably due to the relatively long mixing times required for the experiment. In this paper, we describe the application of a pulsed method that accomplished coherence transfer by isotropic mixing (Braunschweiler & Ernst, 1983) to observe long-range connectivities such as $C^{\alpha}H$–$C^{\delta}H_3$ in proteins.

Materials and Methods

NMR Spectrometer. All NMR experiments were carried out on a home-built 500-MHz NMR spectrometer. The radio-frequency (rf) section is dual-channel design, with each channel based on a PTS-500 synthesizer; 500-MHz pulsed irradiations are produced by mixing a 440-MHz local oscillator with a 60-MHz intermediate frequency. The intermediate frequency is derived from the 10-MHz reference from the PTS-500 synthesizer. Each 60-MHz channel is split into four parallel channels, the phases of which are in quadrature. Each parallel channel consists of an rf switch and continuous phase and amplitude adjusts. The 500-MHz power amplifier is an ENI-525LA, which outputs just over 25 W. The 500-MHz proton probe and broad-band (75–500-MHz) lock channel were purchased from Cryomagnet Systems. The console is also equipped with a small-angle rf phase shifter.

The data system is based on a MICRO-VAX II computer equipped with a 350-Mbyte sealed disk and networked to a second MICRO-VAX II and VAX 11/780. The pulse programmer and digital acquisition processor (DAP) were designed and constructed at the University of Washington. The pulse programmer is an 80 bit wide by 4096 deep random access memory equipped with 64 nestable hardware loops. Each loop may count to 2^{16}. Within a 80-bit pulse programmer word, 32 bits are dedicated to timing, and 32 bits control various console functions.

The DAP is basically a programmable averaging oscilloscope that may acquire, average, and store in dual-channel mode a free induction decay up to 16 384 bits in length. The DAP may acquire data with two 16-bit ADC at rates up to

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‡ Present address: Department of Biochemistry, SJ-70, University of Washington, Seattle, WA 98195.

[1] Abbreviations: HPr, histidine-containing protein; COSY, two-dimensional *J*-correlated spectroscopy; NOESY, two-dimensional NOE spectroscopy; NOE, nuclear Overhauser effect; RELAY, two-dimensional relayed coherence transfer spectroscopy; DAP, digital acquisition processor; PTS, Program Test Sources; ADC, analog-to-digital converter.

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400 KHz or with two 12-bit ADC at rates up to 1 MHz.

The pulse programmer is capable of executing an entire two-dimensional experiment, and because the DAP is equipped with a larger shared memory (64 bits wide, 16 384 deep), the MICRO-VAX II need only intervene at the conclusion of an acquisition. The integrity of the MICRO-VAX II as a multiuser system is maintained by executing spectrometer control as a detached process. Data acquisition software for controlling the DAP and pulse programmer was developed at the University of Washington. Data processing software was obtained from Infinity Data Systems. Details on console design will appear elsewhere (J. Gladden and G. Drobny, unpublished results).

Sample Preparation. HPr samples were prepared as described in the preceding paper (Klevit et al., 1986).

NMR Spectroscopy. Coherence transfers within large systems of scalar-coupled spins were accomplished by multiple pulse trains applied during the mixing period of a two-dimensional experiment. An MLEV-16 sequence, as suggested by Davis and Bax (1985), was employed, but other sequences such as WALTZ-16 (Shaka et al., 1983) have also proven useful on other protein samples.

Pulse powers of up to 25 W were applied for mixing periods as long as 70 ms without apparent damage to probes or sample. Up to 300 t_1 values were gathered in each experiment, and sine bell apodization was applied in both t_1 and t_2 dimensions. Absolute magnitude transforms were calculated.

Results and Discussion

Coherence Transfer by Isotropic Mixing. In the homonuclear shift-correlated experiment (COSY), a transfer of coherence is accomplished by the application of a nonselective pulsed irradiation to a system of coherently coupled spins at the conclusion of a free-precession period. In the weak coupling limit, i.e.

$$\nu_A - \nu_B = \delta_{AB} \gg J_{AB}$$

the spin–spin coupling may be treated as a perturbation, and transition frequencies may be defined in terms of eigenvalues of a single spin operator, the chemical shift. In this limit, coherent correlations, indicated by cross peaks in the two-dimensional spectrum, may only be observed between the coherent states of *directly* coupled spins.

The RELAY experiment (Eich et al., 1982) allows the observation of coherent correlations between spins that are not directly coupled but that belong to a coupled network. This experiment involves transformations by pulsed irradiations of single quantum states of second rank created during the evolution period (t_1) of a two-dimensional experiment. For example, a single quantum state of spin A that is antiphase with respect to spin M is converted by a nonselective pulse to M magnetization that is antiphase with respect to A. Subsequent evolution during the mixing period due to coupling between A and M, and M and X, eventually produces M magnetization that is an antiphase with respect to X, and a final pulsed irradiation converts this coherent state into X magnetization that is antiphase with respect to M. The latter coherent state evolves under the spin-interaction Hamiltonian during the detection period and is amplitude-modulated by the A chemical shift and AM coupling interaction as a result of evolution during t_1. This modulation results in a cross peak between A and X in the two-dimensional spectrum even though A and X are not directly coupled. In general, each relay step requires a nonselective pulse and a mixing period, the length of which is adjusted to maximize the relayed coherence transfer (Bax & Drobny, 1985).

The situation changes however when the spin system is strongly coupled:

$$\delta_{AB} \simeq 0\ (J_{AB})$$

In this case, it is possible to observe cross peaks between nuclei that are not directly coupled. For example, in a strongly coupled ABC spin system, all possible cross peaks may be observed even when $J_{AC} = 0$. This occurs because the evolution of the spin system may no longer be described in terms of single-spin isochromats, a fact that is obvious when one considers that when δ_{AB} and J_{AB} become comparable, spectral transition frequencies no longer occur simply at the chemical shifts of spins A, B, and C.

The experimental methods used in this work are based upon the principle of coherence transfer by isotropic mixing. Imagine a system of three weakly coupled spins: A, M, and X, where $J_{AX} = 0$ but J_{AM} and J_{MX} are nonzero. Transverse magnetization is produced by a nonselective 90° pulse applied to the A, M, and X spins. Subsequent development of the spin system may be described in terms of the precession of A, M, and X isochromats in the transverse plane under the influence of the various chemical shift and spin–spin interactions. At the conclusion of the evolution period, coherence transfer is accomplished not by simply applying a nonselective 90° pulse but by removing or reducing the chemical shift interactions so that for each nuclear pair AB in a coherently coupled network $\delta_{AB} \simeq 0\ (J_{AB})$. The result is that during the mixing period the spin-interaction Hamiltonian assumes a strong coupling form, and in accordance with our remarks on strongly coupled spin systems, coherence transfers occur throughout the coupled network. Thus, it is possible with such a pulsed method to observe long-range coherent correlations in weakly coupled spin systems. Müller and Ernst (1979) have described how complete transfer of coherence may be accomplished between heteronuclei by a spin-locking sequence. When the Hartmann–Hahn (Hartmann & Hahn, 1962) condition is fulfilled, coherence is transferred in an oscillatory fashion between coupled nuclei. Braunschweiler and Ernst (1983) have described several experiments that accomplish coherence transfers by isotropic mixing in coupled spin systems. Collectively named total coherence transfer spectroscopy (TOCSY), these experiments utilize trains of pulses in the mixing period to remove or reduce the Zeeman Hamiltonian by coherent averaging (Haeberlen, 1976). The result is an average-mixing Hamiltonian that is dominated by the isotropic spin–spin coupling

$$H = \sum_{i<j} J_{ij} I_i \cdot I_j$$

that effects coherence transfers throughout coupled spin networks as described above. Weitekamp et al. (1982) have suggested a similar pulse sequence appropriate for use in solids and liquid crystals. Finally, Bax and co-workers have produced isotropic-mixing Hamiltonians through the use of the MLEV-16 sequence (Davis & Bax, 1985) and have dubbed this pulse sequence HOHAHA.

In fact, isotropic mixing may be accomplished by a variety of pulse sequences. WALTZ-16 (Shaka et al., 1983) may be used to remove the Zeeman terms in the mixing Hamiltonian, and dipolar line-narrowing sequences such as MREV-8 (Rhim et al., 1973) are also appropriate if π pulses are inserted into the cycle. Figure 1 illustrates the general pulse scheme used in this work.

Illustrative Example: AX Spin System. As we mentioned above, our objective is to reduce or remove the Zeeman terms in the spin-interaction Hamiltonian during the mixing period.

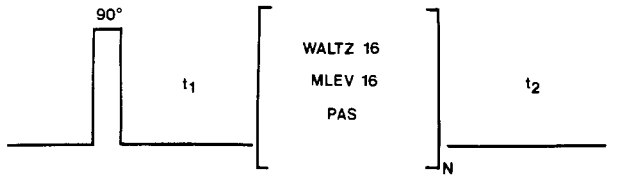

FIGURE 1: Coherence transfer by isotropic mixing may be accomplished by applying a variety of multiple phase sequences during the mixing period. Examples include WALTZ-16, MLEV-16, and a train of phase-alternated 180° pulses (PAS). WALTZ-16 is given by the sequence $Q\bar{Q}\bar{Q}Q$, where $Q = 270°(-\chi)360°(\chi) + 80°(-\chi)270°(\chi)$-$90°(-\chi)180°(\chi)360°(-\chi)180°(\chi)270°(-\chi)$. MLEV-16 is given by the sequence $RRR\bar{R}\ \bar{R}RR\bar{R}\ \bar{R}\bar{R}RR\ R\bar{R}\bar{R}R$, where $R = 90°(\chi)$-$240°(\chi)90°(\chi)$

Now the duration of the mixing irradiation (or the number of pulse cycles) required to observe a correlation depends on the properties of the spin system. This means that to determine the exact duration of irradiation during the mixing period a knowledge of the number of spins within the coupled network, their spatial arrangement, and the magnitude of the spin–spin couplings is required. Such considerations have lead to an optimization of relayed coherence transfers in weakly coupled spin systems (Bax & Drobny, 1985), and we seek a similar optimization in the present case.

A quantitative treatment of isotropic mixing in multispin systems will be addressed in another place. For the present, it suffices to illustrate the dependence of cross-peak intensity on length of the mixing irradiation by a simple example: an AB system subjected to a continuous rf irradiation during the mixing period. This problem has been treated for the heteronuclear case (Müller & Ernst, 1979).

It may be shown that for such experiments the NMR signal as a function of the evolution time t_1 and the detection time t_2 is given by

$$S_y(t_1,t_2) = \sum_{ijkl}(S_y(t_1,t_2))_{ijkl} = \sum_{ijkl} e^{-i\Omega_{jk}t_1}e^{-i\Omega_{il}t_2}P_{ij}Q_{kl}(F_y)_{jk}(F_y)_{li}$$

where $(S_y)_{ijkl}$ corresponds to a transfer of coherence from a coherent superposition of state j and k to a coherent superposition of states i and l. $\Omega_{ij} = \omega_i - \omega_j$ represents the four transition frequencies of the spin system, and $\{\omega_i\}i = 1,4$ is the set of eigenvalues of the spin system:

$$\omega_1 = \frac{-1}{2}(\omega_A + \omega_B) - \frac{J}{4}$$

$$\omega_2 = \frac{1}{2}\left(\frac{J}{2} + R\right)$$

$$\omega_3 = \frac{1}{2}\left(\frac{J}{2} - R\right)$$

$$\omega_4 = \frac{1}{2}(\omega_A + \omega_B) - \frac{J}{4}$$

where $R = (\delta^2 + J^2)^{1/2}$ and $\delta = (\omega_A - \omega_B)$.

The signal intensity is defined by the product of four matrix elements: $P_{ij}Q_{kl}(F_y)_{jk}(F_y)_{li}$. F_y is the spin angular momentum operator I_y, expressed in the eigenbasis of the spin-interaction Hamiltonian. P and Q are coherence transfer matrices and represent the action of the rf irradiation applied during the mixing period.

As an example, consider the cross peak that occurs at the frequency coordinate $(\Omega_{21}, \Omega_{43}) = ({}^1/_2[\omega_A + \omega_B + J + R], {}^1/_2[\omega_A + \omega_B - J + R])$. The relevant coherence transfer matrix elements are P_{42} and Q_{13}, which have the form

$$P_{42} = P_1e^{-i\Omega_{M1}\tau_M} + P_2e^{-i\Omega_{M2}\tau_M} + P_3e^{-i\Omega_{M3}\tau_M} + P_4e^{-i\Omega_{M4}\tau_M}$$

$$Q_{13} = Q_1e^{+i\Omega_{M1}\tau_M} + Q_2e^{+i\Omega_{M2}\tau_M} + Q_3e^{+i\Omega_{M3}\tau_M} + Q_4e^{+i\Omega_{M4}\tau_M}$$

The coefficients $\{P_i\}$ and $\{Q_i\}$ are dependent on the rf field strength, chemical shifts, and scalar couplings. Their forms are complicated and will not be considered here. $\{\Omega_{M_i}\}$ is the set of eigenvalues of the mixing Hamiltonian and in the limit of strong rf field assumes the form

$$\omega_{M_1} = -\frac{1}{2}[(\omega_A^2 + \omega_1^2)^{1/2} + (\omega_B^2 + \omega_1^2)^{1/2}] - \frac{J}{4}$$

$$\omega_{M_2} = \frac{1}{2}\left(\frac{J}{2} + S\right)$$

$$\omega_{M_3} = \frac{1}{2}\left(\frac{J}{2} - S\right)$$

$$\omega_{M_4} = \frac{1}{2}[(\omega_A^2 + \omega_1^2)^{1/2} + (\omega_B^2 + \omega_1^2)^{1/2}] - \frac{J}{4}$$

where $S = (\Delta^2 + J^2)^{1/2}$ and $\Delta = (\omega_A^2 + \omega_1^2)^{1/2} + (\omega_B^2 + \omega_1^2)^{1/2}$.

As Müller and Ernst have observed (Müller & Ernst, 1979), the spectrum of the coherence transer process is composed of six frequencies obtained in the present case by multiplying Q_{13} and P_{42}. This means that the expression for the cross-peak intensities will contain terms of the form $e^{i(\Omega_j - \Omega_k)\tau_M}$, and hence, the cross-peak amplitudes will oscillate as a function of τ_M.

Of particular interest is the mode $\Omega_{M_2} - \Omega_{M_3}$, which corresponds to the exchange of coherence parallel to the effective field during the mixing period (i.e., spin-locked magnetization). In the limit of strong rf field, this mode produces a $\sin^2(\pi J\tau_M)$ dependence of the cross-peak amplitude. Therefore, the cross-peak intensity will oscillate as a function of τ with maxima occurring at $(n + 1)/(2J)$.

Application of Technique to HPr. Figure 2 shows the spectrum obtained by applying an MLEV-16 sequence during the mixing period of a two-dimensional experiment to a sample of HPr in D_2O. A total of 80 MLEV-16 cycles was applied for a total irradiation of about 70 ms. The resulting spectrum contains many more cross peaks than a COSY spectrum of the same sample (Figure 2, top). The new cross peaks can be categorized into three groups: (1) connectivities between weakly coupled $C^\alpha H$–$C^\beta H$ pairs that do not appear (or appear very weakly) in a normal COSY spectrum, (2) connectivities between $C^\alpha H$–$C^\gamma H$ pairs that also appear in optimized RELAY spectra, and (3) new long-range connectivities.

The COSY cross peaks of Gln-3, Glu-5, and Glu-66 are representative of the first type of cross peaks. These cross peaks are just barely visible in a COSY spectrum. Furthermore, the MLEV-16 spectrum contains cross peaks for both of the C^β-protons for these and other side chains. Quite often, a COSY spectrum contains only one of the two expected cross peaks. The second $C^\beta H$ resonance must then be found by looking for a strong NOESY peak (since both $C^\beta Hs$ will be close to the $C^\alpha H$) and confirming the assignment via the strong $C^\beta H$–$C^{\beta'}H$ COSY cross peaks. Another COSY-type connectivity appears in the MLEV-16 spectrum of HPr but not in a COSY spectrum derived from a proline spin system (cross peaks contained in boxes in Figure 2, bottom). The presence of the prolyl resonances in the upper box, corresponding to $C^{\gamma,\gamma'}H$–$C^{\gamma,\gamma'}H$, had been deduced on the basis of interresidue NOEs and is now confirmed by the current experiment.

$C^\alpha H$-to-$C^\gamma H$ cross peaks that can be observed in a standard RELAY spectrum are very strong in an MLEV-16 spectrum. An optimized RELAY spectrum for HPr [see Figure 2 in Klevit et al. (1986)] contains $C^\alpha H$-to-$C^\gamma H$ cross peaks for five

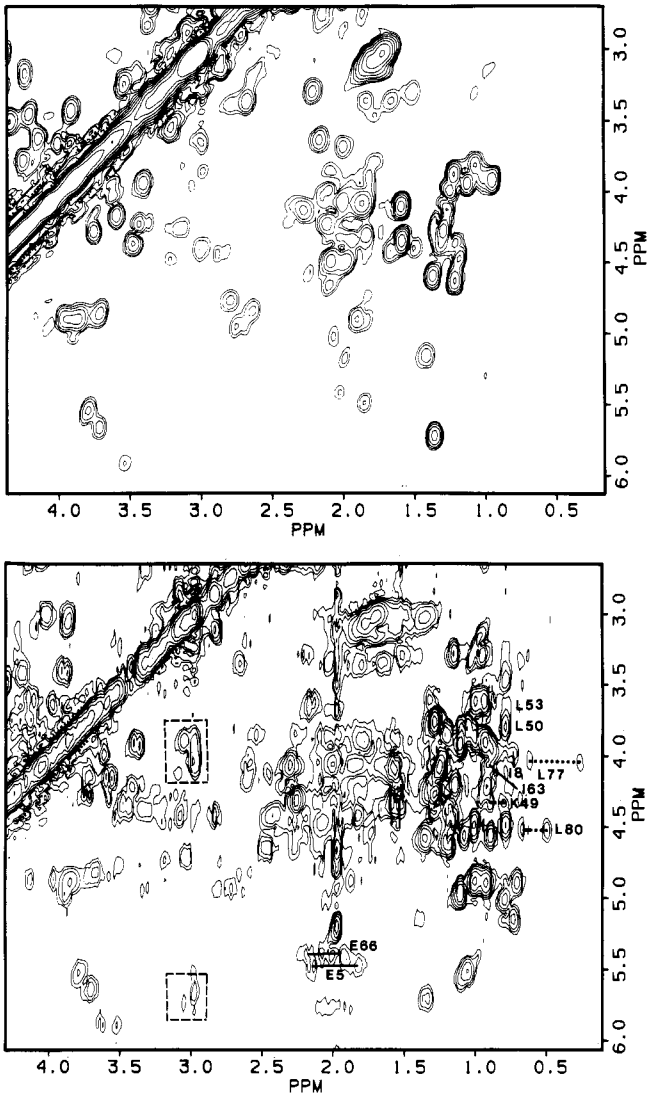

FIGURE 2: Comparison of COSY and MLEV-16 spectra of HPr in D_2O. (Top) The region of a COSY spectrum that contains the $C^{\alpha}H$–$C^{\beta}H$ cross peaks. This spectrum was obtained under conditions identical with those of the spectrum below (spectral width 10 000 Hz; 64 scans per t_1 experiment; 300 t_1 experiments; digital resolution 9.8 Hz/point; 30 °C). (Bottom) Identical region of spectrum obtained by applying 80 MLEV-16 cycles during the mixing time. Several features of the spectrum are pointed out in the figure: the $C^{\alpha}H$–$C^{\beta}H$, $C^{\alpha}H$–$C^{\beta\prime}H$ pairs of E5 and E66 are indicated; cross peaks of a proline spin system are shown in boxes (the lower box contains $C^{\alpha}H$–$C^{\gamma,\gamma\prime}H$ and the upper box contains $C^{\gamma,\gamma\prime}H$–$C^{\delta,\delta\prime}H$); the long-range $C^{\alpha}H$–$C^{\delta,\delta\prime}H$ connectivities for four leucine spin systems are labeled.

threonines, seven valines, two isoleucines, and a number of longer side chains such as methionine and glutamine. These all appear in the current spectrum.

The remaining cross peaks in the spectrum represent new long-range intraresidue connectivities. There are both single-step connectivities (e.g., $C^{\alpha}H$–$C^{\gamma}H$, $C^{\beta}H$–$C^{\delta}H$, $C^{\gamma}H$–$C^{\epsilon}H$) and double-step connectivities ($C^{\alpha}H$–$C^{\delta}H$). An example of a new single-step $C^{\alpha}H$–$C^{\gamma}H$ connectivity is that of Ile-63, which did not appear in the conventional RELAY spectrum. Another example is the $C^{\alpha}H$–$C^{\gamma,\gamma\prime}H$ cross peaks for Pro-11, contained in the bottom box in Figure 2 (bottom). Single-step $C^{\beta}H$–$C^{\delta}H_3$ connectivities for leucines were also observed. Since the sequential assignment method gives assignments through $C^{\beta}H$ resonances, these new cross peaks can be used to assign the methyl resonances to specific leucyl side chains.

Double-step $C^{\alpha}H$–$C^{\delta}H_3$ connectivities are observed at a level well above noise for four leucines and one isoleucine in the HPr MLEV-16 spectrum. On the basis of the sequential assignments of HPr, the residues were identified as Leu-50, Leu-53, Leu-77, Leu-80, and Ile-8. A series of spectra of HPr have been obtained with a variety of mixing times corresponding to 40, 50, 60, 70, and 90 MLEV-16 cycles. Although peak intensities varied, no new cross peaks were observed with either shorter or longer mixing times. Work is now in progress to determine quantitatively the build-up of long-range connectivities in large spin systems (J. Listerud and G. Drobny, unpublished results). We have performed long-range coherence transfer experiments utilizing MLEV-16 and WALTZ-16 sequence on a variety of proteins, ranging in size from ~7 kDa to ~12 kDa, and in every case have obtained strong long-range connectivities that provide side-chain resonance assignments that will ultimately provide useful structural information.

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