^{1}H NMR Studies of λ Cro Repressor. 1. Selective Optimization of Two-Dimensional Relayed Coherence Transfer Spectroscopy†

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ABSTRACT: Two-dimensional relayed coherence transfer NMR spectroscopy (RELAY) has been used to corroborate side chain spin system identities in crowded regions of the ^{1}H NMR spectrum of the λ cro repressor protein. The mixing time in the RELAY experiments was optimized for specific preselected spin systems by using recently developed methods [Bax, A., & Drobny, G. (1985) *J. Magn. Reson. 61*, 306–320], which utilize the transverse relaxation time (T_2) of the molecule and relevant J couplings for the defined spin system. We demonstrate that a mixing time of 26 ms gives rise to strong $C^{\alpha}H$–$C^{\gamma}H_3$ RELAY cross peaks for all valine, threonine, and isoleucine residues, while RELAY cross peaks for other spin systems are weak or are not observed. This allows for rapid and unambiguous identification of the side chain resonances for valine, isoleucine, threonine, and alanine (by elimination). The use of optimized RELAY for analyzing and identifying spin systems in complex spectra is discussed.

The relayed coherence transfer (RELAY)[1] experiment (Bolton, 1982; Eich et al., 1982) is a powerful method for analyzing spin systems with poorly resolved NMR spectra. The RELAY experiment is essentially an extended COSY, where the second 90° COSY pulse has been replaced with a $90°-\tau/2-180°-\tau/2-90°$ pulse train. For an AMX system, this effects the transfer of I_AI_M coherence to spin X; the 2D RELAY spectrum will then contain an AX cross peak, although the two spins are not directly coupled. The method is particularly useful in unambiguously identifying spin systems with overlapping resonances, which would otherwise be impossible using the conventional COSY experiment.

The application of RELAY to proteins has been previously reported (King & Wright, 1983; Wagner, 1983; Zuiderweg et al., 1983), but the results obtained were suboptimal due to the use of incorrect mixing times τ in these experiments. Until now the evolution of the relayed magnetization during the mixing time has not been subjected to rigorous treatment. Bax & Drobny (1985) have recently analyzed the RELAY experiment for several different spin systems and have shown that the efficiency of the relayed magnetization is highly dependent on the type of spin system involved. Given the general usefulness of the RELAY experiment for assigning proteins, we have improved the effectiveness of the experiment using the guidelines of Bax & Drobny (1985). These improvements include (i) using mixing times that optimize the magnetization transfer between specific, predetermined spin systems and (ii) using a pulse phase cycle that allows the experiment to be carried out on-resonance, thus doubling the resolution in the ω_2 dimension compared to that for the off-resonance experiment. The phase cycling also eliminates NOE cross peaks.

In this paper we report our findings for optimized RELAY experiments on the λ cro repressor system. The X-ray structure of cro has been reported (Anderson et al., 1981) and suggests a possible mode of binding to B-DNA (Ohlendorf et al., 1982). The small size of cro (66 amino acids, dimer M_r 14 700) makes it an attractive NMR model for studying protein–DNA interactions in solution. The O_R3 operator DNA to which cro binds has been synthesized and its NMR spectrum completely assigned (Wemmer et al., 1984). Up to now work on the complex has been hindered by a complete lack of sequential resonance assignments of cro. The RELAY experiments reported here are an essential step in obtaining such assignments,[2] which are reported in the following paper (Weber et al., 1985).

Materials and Methods

Cro Repressor Purification. Cro was isolated from *Escherichia coli* RB791/pCL1 cells, which produce large quantities of plasmid-encoded cro protein when induced with isopropyl β-D-thiogalactoside (IPTG) (Tom Roberts, personal communication). A typical preparation started with approximately 400 g of frozen cells (the yield of a 100-L culture). All steps were done at 4 °C. Cells were homogenized in a Waring blender at low speed in 500 mL of 10% sucrose, 50 mM Tris-HCl, pH 6.4, 1 mM EDTA, and 50 mM KCl, with fresh PMSF added. Cells were disrupted either by sonication or by addition of lysozyme (0.2 g/L final concentration) and 1–5 mg of DNase I. Potassium chloride was added to a final concentration of 0.5 M, and the suspension was centrifuged to remove cell debris. The pellet from a 25–70% saturated ammonium sulfate cut was dissolved in 500 mL of 10 mM potassium phosphate, pH 6.4, 1 mM EDTA, 0.1 M KCl, and

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[1] Abbreviations: RELAY, two-dimensional relayed coherence transfer NMR spectroscopy; COSY, two-dimensional J-correlated NMR spectroscopy; NOE, nuclear Overhauser effect; $J_{N\alpha}$, $^3J_{NH\text{-}C^{\alpha}H}$; $J_{\alpha\beta}$, $^3J_{C^{\alpha}H\text{-}C^{\beta}H}$; $J_{\beta\gamma}$, $^3J_{C^{\beta}H\text{-}C^{\gamma}H}$; 2D, two dimensional; FID, free induction decay; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

[2] Throughout this paper, we shall use the following convention: an *assignment* refers to the correlation between a resonance in the spectrum with a particular proton on the protein, while an *identification* is to a *type* of amino acid. Hence side chains are *identified* in this paper as valines, threonines, etc. but are *assigned* in the following paper to a particular valine, or threonine, on the molecule.

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Table I: Pulse Phases in the RELAY Experiment[a]

step	ϕ_1	ϕ_2	receiver
1	x	x	+
2	x	$-x$	+
3	y	y	-
4	y	$-y$	-
5	$-x$	$-x$	+
6	$-x$	x	+
7	$-y$	$-y$	-
8	$-y$	y	-

[a] This sequence was repeated four times, with all pulse and receiver phases incremented 90° each time.

5% (v/v) glycerol (buffer A) and added batchwise to 100 g of phosphocellulose (Whatman P11) precycled and preequilibrated in buffer A. The resin was washed with buffer A + 0.1 M KCl, buffer A + 0.25 M KCl, and buffer A + 1.0 M KCl. The last filtrate was saved and concentrated by addition of ammonium sulfate (final concentration, 60% saturated). Cro was then purified by phosphocellulose and Sephadex G-50 chromatography (Folkmanis et al., 1976). The initial phosphocellulose batch elution was used to avoid use of larger phosphocellulose columns, which often clogged during sample loading.

NMR. The NMR sample concentration of cro was 3 mM (monomer) in 0.36 mL of 10 mM potassium phosphate, pH 4.6, containing 0.2 M KCl and 0.1 mM EDTA; alternatively, cro was dissolved in 10 mM potassium phosphate, pH 6.8, without added salt. Samples were lyophilized and were redissolved twice in 0.36 mL of 99.996% D_2O (Stohler Isotope Chemicals) for the nonexchangeable proton spectra. Reported chemical shifts are relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).

The RELAY experiments used the pulse sequence

$$90^\circ_x\text{–}t_1\text{–}90^\circ_{\phi_1}\text{–}\tau/2\text{–}180^\circ_{\phi_1}\text{–}\tau/2\text{–}90^\circ_{\phi_2}\text{–}t_2$$

and the phase cycle shown in Table I (Bax & Drobny, 1985). This phase cycling scheme allows the experiment to be performed on-resonance and suppresses the NOE peaks (Bax & Drobny, 1985). The mixing time τ was optimized for different spin systems (see below). Spectra were obtained at 35 °C on a Bruker WM500 spectrometer using a spectral width of 6024.1 Hz by collecting 1024 points in t_2 and using quadrature detection. The carrier was placed on the solvent resonance in the center of the spectrum. Approximately 400 t_1 experiments were obtained unless noted otherwise. Each t_1 experiment collected 64 scans.

The data were copied onto magnetic tape and transferred to a VAX 11/780 for processing using software developed by Dr. Dennis Hare (unpublished program). The data in each dimension prior to transformation were multiplied by a non-phase-shifted sine bell function adjusted to the end of the data and skewed toward the beginning of the FID to match the peak of the RELAY signal. After transformation, the first 50 or last 20 rows of the 2D matrix contained no resonances and were averaged; this dummy "row" was then subtracted from all rows in the matrix (Klevit, 1985). The net effect was to minimize t_1 streaking without noticeably affecting the cross peaks in the spectra.

RELAY Optimization. An optimal mixing time produces (i) maximization of relayed magnetization between spins and/or (ii) minimization of COSY and diagonal peaks. Since RELAY peaks are usually weaker than COSY peaks, it is more important to ensure that the first of these two considerations is met. Accordingly, all reported optimizations have been calculated to obtain maximal RELAY intensities. The

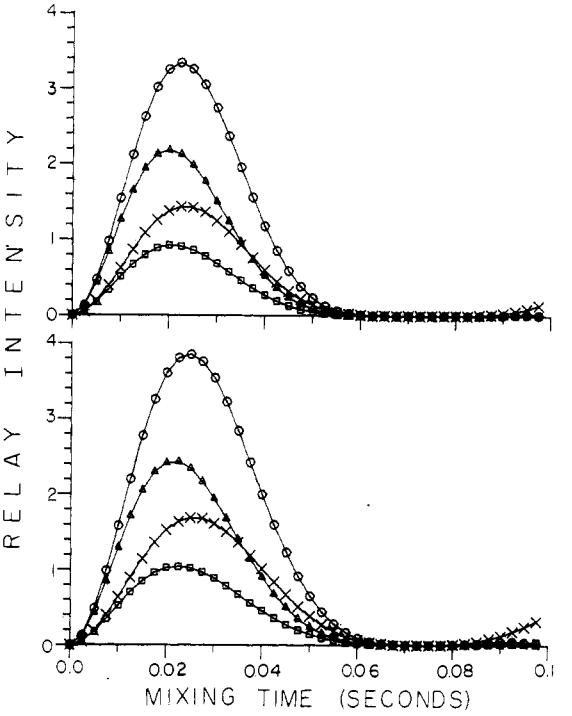

FIGURE 1: Relative RELAY intensities for AMX_6 (top) and AMY_3X_3 (bottom) systems as a function of mixing time. An intensity of 1 corresponds to the predicted intensity of an AX system where $J_{AX} = (J_{AM} + J_{MX})/2$ in a regular COSY experiment using identical acquisition parameters. For both spin systems, the following designations were used: (○, △) J_{AM} = 10 Hz; (×, □) J_{AM} = 4 Hz; (○, ×) T_2 = 50 ms; (△, □) T_2 = 25 ms. In all calculations, $J_{MX} = J_{MY}$ = 6.9 Hz.

time dependences of the relayed magnetization for the spin systems investigated are

$$I_{AMX} = [\sin(\pi J_{AM}\tau)\sin(\pi J_{MX}\tau)]e^{-\tau/T_2}$$

$$I_{AMXX'} = [\sin(\pi J_{AM}\tau)][\sin(\pi J_{MX'}\tau)\cos(\pi J_{MX}\tau) + \sin(\pi J_{MX}\tau)\cos(\pi J_{MX'}\tau)]e^{-\tau/T_2}$$

$$I_{AMX_3} = [3\sin(\pi J_{AM}\tau)][\sin(\pi J_{MX}\tau) + \sin(3\pi J_{MX}\tau)]e^{-\tau/T_2}$$

$$I_{AMQX} = [\sin(\pi J_{AM}\tau)\sin(\pi J_{MX}\tau)\cos(\pi J_{MQ}\tau)]e^{-\tau/T_2}$$

$$I_{AMX_6} = [\sin(\pi J_{AM}\tau)] \times [5\sin(2\pi J_{MX}\tau) + 4\sin(4\pi J_{MX}\tau) + \sin(6\pi J_{MX}\tau)]e^{-\tau/T_2}$$

$$I_{AMY_3X_3} = [\sin(\pi J_{AM}\tau)][\sin(3\pi J_{MY}\tau) + 5\sin(\pi J_{MY}\tau)\cos(3\pi J_{MX}\tau) + 3\cos(\pi J_{MX}\tau)]e^{-\tau/T_2}$$

$$I_{AMPQX_3} = [\sin(\pi J_{AM}\tau)\cos(\pi J_{MP}\tau)\cos(\pi J_{MQ}\tau)] \times [\sin(\pi J_{MX}\tau) + \sin(3\pi J_{MX}\tau)]e^{-\tau/T_2}$$

where in each case magnetization from spin A is relayed to spin X (Bax & Drobny, 1985).

A RELAY experiment was designed to help in identifying the side chain spin systems for the three valine, six threonine, and five isoleucine residues in cro. The spin systems for these residues are similar, being AMX_3 (Thr), $AMQPX_3$ (Ile), and AMX_6 or AMY_3X_3 (Val, where the AMX_6 system is appropriate if the chemical shifts of the two methyl groups are equivalent). In each case magnetization from the α proton is relayed through a single β proton to the γ methyl group(s). Not surprisingly, the optimal mixing time for each is very similar, near 26 ms for a molecule with T_2 = 25 ms. Figure 1 shows representative calculations for the two possible valine systems, varying the $J_{\alpha\beta}$ couplings between 4 and 10 Hz; the $J_{\beta\gamma}$ couplings in valine are fixed at 6.9 Hz (Bundi & Wüthrich, 1979). In all calculations (including those below) the trans-

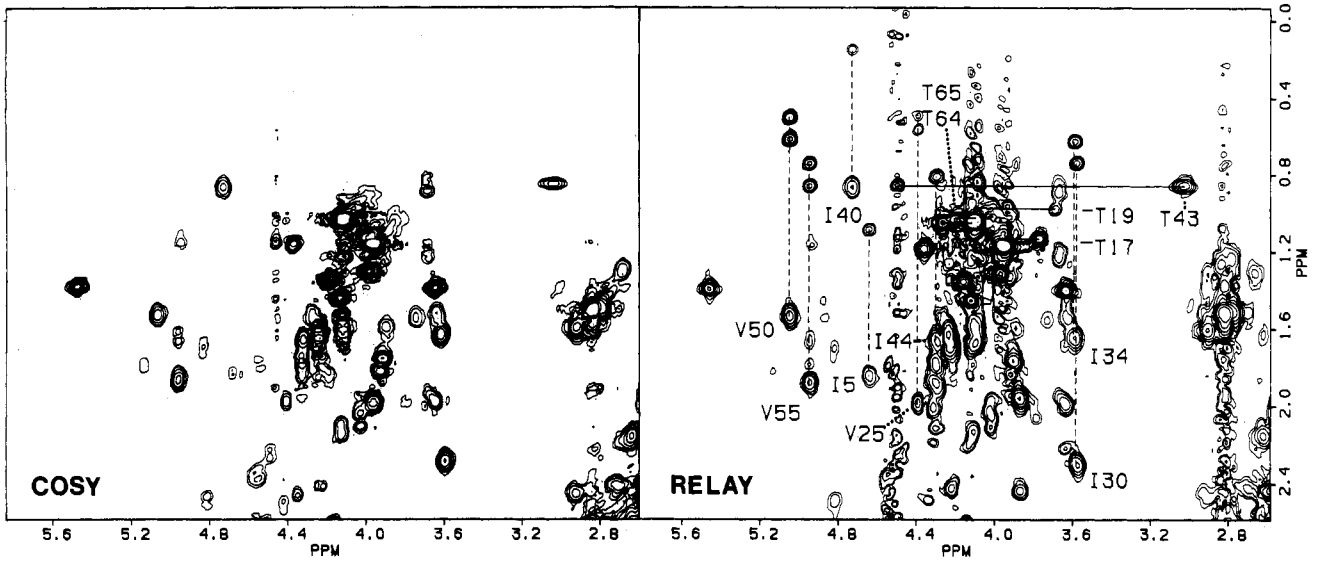

FIGURE 2: Contour plots of 500-MHz COSY and RELAY spectra of cro repressor in D_2O, showing the same expanded region. In the RELAY spectrum, connectivities between the $C^{\alpha}H$–$C^{\beta}H$ COSY cross peaks and the $C^{\alpha}H$–$C^{\gamma}H_3$ RELAY cross peaks for the three valines and the five isoleucines are connected by broken lines and labeled. In addition, the $C^{\beta}H$–$C^{\gamma}H_3$ COSY cross peaks are connected by solid lines to the $C^{\alpha}H$–$C^{\gamma}H_3$ RELAY cross peaks for five of the six threonines, which are also labeled. The RELAY cross peak for Thr-6 cannot be clearly seen, due to overlap with the alanine $C^{\alpha}H$–$C^{\beta}H_3$ COSY cross peaks. Each of these three amino acids gives its own distinctive connectivity pattern (see text). The COSY experiment was obtained in 16 h with a spectral width of 5208 Hz. The RELAY experiment used a total mixing time of 26 ms; total acquisition time was 24 h. A relaxation delay time of 2.5 s was included between scans. The residual HDO signal was not irradiated during the RELAY experiment.

verse relaxation time T_2 was varied between 25 and 50 ms. The apparent T_2 was estimated from line widths of well-resolved $C^{\alpha}H$ peaks and from the FID. The effect of shorter T_2 values was to significantly reduce the relay intensity but only shift the optimal mixing time to slightly shorter values. Figure 1 shows the predicted intensities of RELAY cross peaks as a function of mixing time for different $J_{\alpha\beta}$ and T_2 values in both AMX_6 and AMY_3X_3 systems. Note that varying $J_{\alpha\beta}$ results only in a change of relay intensity and that an AMY_3X_3 system has an optimal mixing time at a slightly longer value than for an AMX_6 system. The experimental mixing time of 26 ms was chosen to compensate for the occurrence of AMY_3X_3 systems; in addition, $C^{\alpha}H$–$C^{\gamma}H_3$ relays of isoleucine and threonine will also be very near an optimum at this mixing time.

Results and Discussion

Figure 2 shows the appropriate regions of the COSY and RELAY spectra of cro repressor in D_2O. Assignments for the indicated cross peaks were obtained by using sequential assignment strategies (Wüthrich et al., 1982; Billeter et al., 1982) and are presented in the following paper in this issue (Weber et al., 1985). As predicted, RELAY cross peaks are observed for all valine, threonine, and isoleucine residues. The five isoleucine residues and six threonine residues exhibit strong $C^{\alpha}H$–$C^{\gamma}H_3$ RELAY cross peaks, although the RELAY cross peak for Thr-6 is in a very crowded spectral region and is not clear in this figure. The four cross peaks corresponding to the Val-50 and Val-55 $C^{\alpha}H$–$C^{\gamma}H_3$ relays are strong, while those for Val-25 are relatively weak. The reason for the weaker Val-25 cross peaks is discussed below. In addition, there is some asymmetry apparent between the two $C^{\alpha}H$–$C^{\gamma}H_3$ relays of the valines. The reason for this asymmetry is not known, although it has also been seen in the *lac* headpiece (Zuiderweg et al., 1983).

This RELAY experiment was designed to aid in identifying the side chain resonances of the protein; apparent in Figure 2 is that the distinctive patterns of the RELAY and COSY cross peaks for these three residues (Val, Ile, Thr) make them easily identifiable. In addition, by identification of the threonine $C^{\alpha}H$–$C^{\gamma}H_3$ cross peaks, the eight alanines (which give $C^{\alpha}H$–$C^{\beta}H_3$ COSY cross peaks of similar intensity and in the same region of the spectrum) may be identified by elimination. Complete identification of the isoleucine side chains requires the further identification of the $C^{\gamma 1}H_2$ and $C^{\delta}H_3$ resonances, which may not always be possible but is certainly simplified by this method. In sum, a full one-third of the side chains in cro have been *readily* and *unambiguously* identified as to their amino acid type; this is a vital step for the sequential assignments. The ease of identifying these systems is also aided by the lack of strong RELAY cross peaks that occur at this mixing time (26 ms). A few other RELAY peaks are seen in Figure 2; the only strong one occurs for the $C^{\alpha}H$–$C^{\gamma}H_2$ RELAY of Met-1. Most likely, this is due to the additional flexibility of the N-terminus (see the following paper in this issue).

Some structural conclusions can also be drawn from the RELAY data. First, two threonine RELAY cross peaks are especially strong and correspond to the C-terminal Thr-64 and Thr-65 residues. The relatively sharp resonances of these two threonines are due to their flexibility in solution, which was first observed in the crystal structure (Anderson et al., 1982; see also the following paper in this issue). Regarding the weaker Val-25 RELAY cross peaks (see above), these would originate from a weaker $J_{\alpha\beta}$ coupling, which is consistent with the $C^{\alpha}H$–$C^{\beta}H$ dihedral angles measured from the crystal structure. The angles in Val-50 and Val-55 are 169° and 179.6°, respectively, corresponding to $J_{\alpha\beta}$ values of 10–12.9 Hz. The angle for Val-25, however, is 65° and corresponds to a $J_{\alpha\beta}$ near 3 Hz (DeMarco et al., 1978). As was shown above, the smaller $J_{\alpha\beta}$ couplings in AMX_6 and AMY_3X_3 systems lower the intensity of the relay peak, but the optimal mixing time is not changed.

For Val-25 the measured RELAY intensities shown in Figure 3 are approximately half of those for Val-50 and Val-55, as predicted by our calculations (see above and Figure 1). Also apparent from Figure 3 is that the RELAY cross peaks are not as strong as the direct COSY cross peaks.

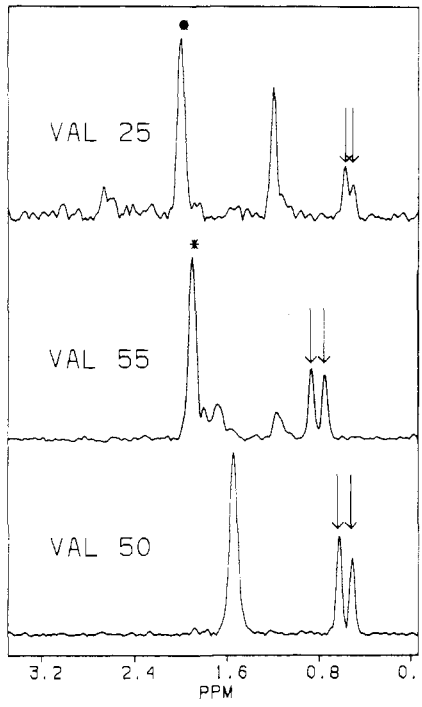

FIGURE 3: Cross sections of the 500-MHz RELAY spectrum through each of the valine α protons. The regions containing the $C^{\alpha}H$–$C^{\beta}H$ COSY cross peak (* for Val-55 and Val-25) and the $C^{\alpha}H$–$C^{\gamma}H_3$ RELAY peaks (arrows) are shown. The valine $C^{\alpha}H$–$C^{\beta}H$ cross peak heights have been normalized to the same value (cf. Figure 2).

Experiments on other proteins in this laboratory have shown that this is chiefly a function of T_2 (unpublished results). The apparent T_2 value used for cro is somewhat short for a symmetric dimer of M_r 14 700; however, aggregation was often a problem, leading to the shorter apparent T_2 value of 25 ms. In any case, the apparent T_2 used in calculating the optimum mixing time for valine relays effected only a slight shift to shorter mixing times. Other considerations are much more important for optimizing the delay period. If one attempts to estimate the optimal mixing time on the basis of only $1/4J$ (King & Wright, 1983; Wagner, 1983; Zuiderweg et al., 1983), one would erroneously deduce that smaller $J_{\alpha\beta}$ couplings would require longer mixing times, which is not the case; in fact, the use of such longer mixing times would produce suboptimal relayed magnetization. King & Wright (1983) mistakenly conclude that valine relays are unobtainable in proteins despite the large $J_{\beta\gamma}$ coupling; however, they report using total mixing times of 60–80 ms, which, from Figure 1, is clearly a null. Our results indicate that $C^{\alpha}H$–$C^{\gamma}H_3$ relays for all valine residues can be obtained in a single experiment and can be optimized regardless of the size of the couplings. The important parameters to consider in optimizing the mixing time are the transverse relaxation time and whether it is an AMX_6 or AMY_3X_3 system.

The method for selectively optimizing RELAY experiments may also be extended to residues other that those described here. The evolution of several of the possible spin systems found in proteins is described in Bax & Drobny (1985), as well as under Materials and Methods (above). Reasonable optimizations can be calculated even in the absence of precise J-coupling and T_2 values, as the position of the maximal relayed intensity often is most dependent upon the type of spin system involved. By varying the input parameters (J and T_2 values) when accurate information is lacking, one may determine a delay time that is a reasonable compromise for several possible combinations of parameters. This is perhaps best done by incorporating the above equations in a short program capable of graphically presenting the evolution behavior of the relayed intensity, following the inputted values. Since the J couplings for amino acids are known, or can be closely approximated when some variability is present, realistic mixing times can usually be calculated. In addition, the dependence of J couplings upon the dihedral angle can be exploited for obtaining NH–$C^{\beta}H$ RELAYs in a particular secondary structure, since the dihedral angles (and J couplings) for β-sheet and α-helical residues are quite different (Ramachandran & Sasesikharan, 1968; Pardi et al., 1984).

This is the first of a planned series of papers describing our NMR studies of the λ phage cro system. The sequential resonance assignments mentioned here are the subject of the following paper in this issue. Finally, we would like to mention our use of the "RELAY" nomenclature: it is a simple name that is descriptive of the experiment being performed. Other names for the experiment have been suggested, which are usually contrived acronyms in the style of Wüthrich and colleagues. We prefer RELAY.

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Registry No. Val, 72-18-4; Thr, 72-19-5; Ile, 73-32-5; Ala, 56-41-7.

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