JOURNAL OF MAGNETIC RESONANCE **128,** 92–97 (1997)
ARTICLE NO. MN971213

Spin-State-Selective Excitation. Application for E.COSY-Type Measurement of J_{HH} Coupling Constants[1]

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Received April 1, 1997; accepted June 30, 1997

A new pulse sequence element, spin-state-selective excitation (S^3E), is introduced and combined with E.COSY-type techniques for measurement of $^1H-^1H$ J coupling constants. S^3E edits the two resonances of a doublet prior to an evolution period of a multidimensional experiment and results in a subspectrum for each resonance. Due to this editing the large heteronuclear one-bond coupling constants normally exploited for separation of submultiplets in E.COSY-type experiments can be suppressed in experiments employing S^3E. Hence there is a concomitant effective increase in resolution. Apart from pulse imperfections and relaxation during a delay $(4J)^{-1}$ S^3E causes no loss of sensitivity in comparison to conventional experiments. Experimental confirmation is done using the protein RAP 17-97 (N-terminal domain of α_2-macroglobulin receptor associated protein). © 1997 Academic Press

Key Words: **E.COSY; multidimensional NMR; J coupling constants; S^3E.**

A convenient way of determining scalar J coupling constants is by generation of multidimensional NMR spectra incorporating the so-called E.COSY procedure (*1–3*). The basic idea of E.COSY is to restrict coherence transfer by a mixing sequence in a multidimensional experiment to occur exclusively between connected transitions. That results in very simple cross peak multiplet structures consisting of basic patterns separated by two-dimensional displacement vectors whose coordinates correspond to J coupling constants. Most importantly, a J coupling constant can be determined accurately irrespective of its size if a displacement vector exists with this J as one of the coordinates provided the other coordinate is well above the linewidth of the resonances.

This stipulation is superbly fulfilled in modern multidimensional NMR of biomolecules typically employing ^{15}N and/or ^{13}C isotopic labeling. The J coupling constants dominating the linewidth are primarily the heteronuclear one-bond J's between a proton and an ^{15}N or ^{13}C nucleus. Hence structurally important $^1H-^1H$ and $^1H-^{15}N$ or $^1H-^{13}C$ J coupling constants can be measured by E.COSY-type techniques (*4–6*).

Consider by way of example a protein enriched with ^{15}N. When no ^{15}N decoupling is applied, the peaks in ^1H-detected 2D experiments may exhibit coupling to ^{15}N. Clearly, if this doubling of the number of peaks could be prevented while still allowing E.COSY-type measurement of J coupling constants, that would be an attractive method of spectral simplification. This Communication describes a novel pulse sequence element to that end and demonstrates how it can be combined with NOESY or TOCSY for measurement of homonuclear coupling constants (in particular $^3J(H^N-H^\alpha)$) by application to the protein RAP 17-97 (N-terminal domain of α_2-macroglobulin receptor associated protein) (*7, 8*).

The goal of halving the number of peaks in the presence of ^{15}N coupling may be achieved by exciting exclusively the amide proton resonances corresponding to the ^{15}N nuclei in either the α or β spin state. Fundamentally, this is very simple to do by a selective pulse but that is obviously not a practical solution with application to biomolecules in mind. First of all the effective sensitivity would be low as the number of separate selective experiments would be double the number of amino acid residues in the protein, and in addition it is very likely that some of the resonances overlap. However, the selective rotation required is an excellent starting point for a systematic derivation of a nonselective pulse sequence having the same effect as the selective rotation (*9–11*).

The systematic strategy starts by the propagator of the required selective rotation and converts it, in a series of steps, to a nonselective pulse sequence. In the present case we have for selective excitation by a y-phase $\pi/2$ pulse of I-spin (^1H) magnetization corresponding to S spins (^{15}N) being in the β state

$$\begin{aligned} e^{i(\pi/2)I_yS^\beta} &= e^{i(\pi/4)(I_y-2I_yS_z)} \\ &= e^{i(\pi/4)I_y}e^{-i(\pi/2)I_x}e^{i(\pi/4)2I_zS_z}e^{i(\pi/2)I_x} \\ &= e^{i(\pi/2)I_x}e^{-i(\pi/2)I_x}e^{i(\pi/4)I_y}e^{i(\pi/2)I_x}e^{i\pi I_x} \\ &\quad \times e^{i\pi S_x}e^{i\pi S_x}e^{i(\pi/4)2I_zS_z}e^{i(\pi/2)I_x} \\ &= e^{i(\pi/4)I_z}e^{i\pi S_x}e^{-i(\pi/4)I_z}e^{i(\pi/2)I_x}e^{i(\pi/4)I_z} \\ &\quad \times e^{iH_0/8J}e^{i\pi(I_x+S_x)}e^{iH_0/8J}e^{i(\pi/2)I_x}, \end{aligned} \quad [1]$$

[1] Presented in part at the 38th ENC, Orlando, Florida, March 23–27, 1997.

1090-7807/97 $25.00

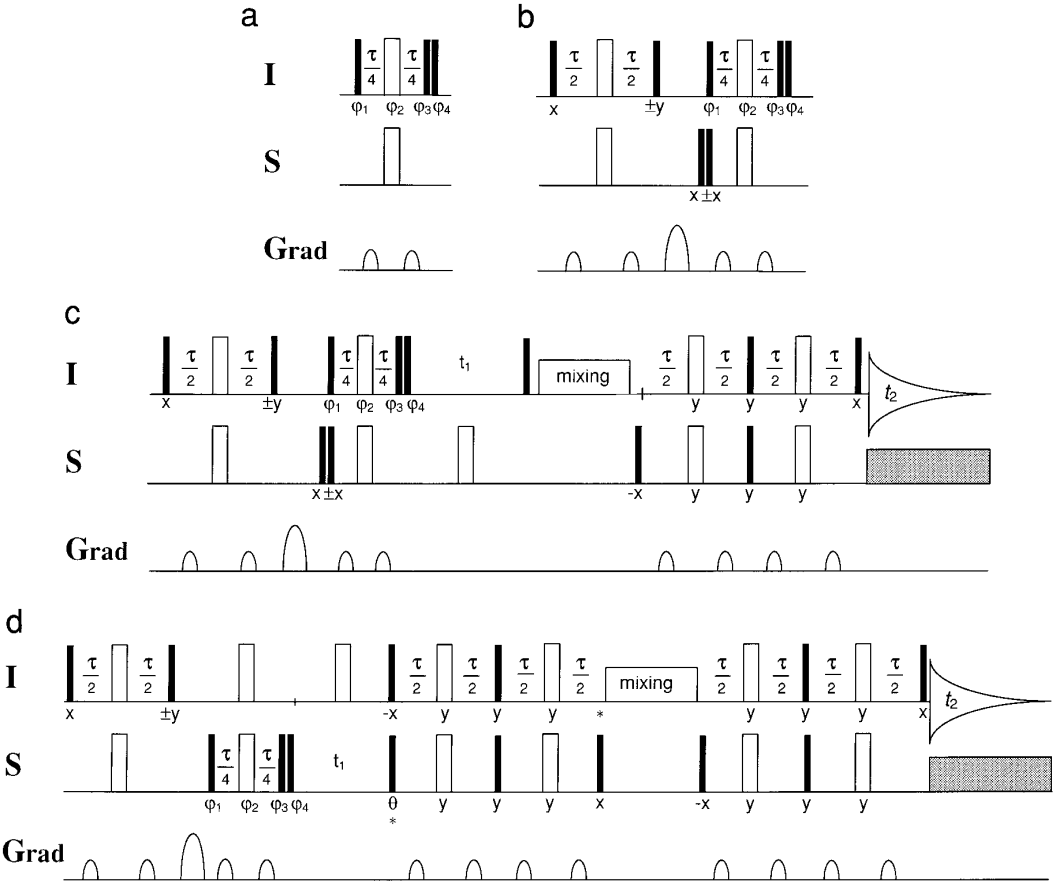

FIG. 1. S^3E pulse sequences for editing of S^α and S^β resonances in I-spin spectra. Filled and open bars represent $\pi/2$ and π pulses, respectively, and phases are indicated below the pulses and the delay τ is $(2J)^{-1}$. The two subspectra are generated by the phase cycles outlined in Table 1. (a) Basic S^3E element; (b) S^3E combined with a preparation element for suppression of magnetizations of I spins not directly attached to S spins; (c) homonuclear and (d) heteronuclear S^3E E.COSY-type pulse sequences for determination of homonuclear coupling constants. The frame designated *mixing* can be either a delay corresponding to NOESY or an isotropic mixing sequence corresponding to TOCSY. In the latter case, the scheme of Cavanagh and Rance (*12*) should be employed while for NOESY we employ the phase cycling of Ref. (*13*). Phases $\pm x$ and $\pm y$ indicate independent two-step phase cycles with opposite receiver phase while $\theta = x$ and $\theta = -x$ is used for echo and antiecho, respectively. In the NOESY variant in (d) the S pulse marked with * is omitted and an I-spin $(\pi/2)_x$ pulse is added at the position of the * on that channel.

where standard operator and propagator transformation formulas (e.g., Ref. (*9*)) have been used and H_0 represents the unperturbed Hamiltonian.

In the final form of Eq. [1] the first two propagators can be ignored when the spin system initially is in a state of thermal equilibrium, and the succeeding three terms represent a $\pi/2$ pulse of phase $\pi/4$.

A pulse sequence equivalent to Eq. [1] and with excitation of resonances corresponding to S spins in the α state can be derived easily but having to perform separate S^α and S^β experiments would reduce the overall sensitivity per unit time by a factor $\sqrt{2}$. Instead another trick can be exploited. As the propagator in Eq. [1] is a $(\pi/2)_y$ rotation on the S^β resonances the magnetization excited will be of phase x and therefore commute with x-phase pulses. Hence the S^α resonances can be excited by a nonselective $\pi/2$ pulse of phase x or $-x$. Coaddition of these two resulting spectra will provide the S^β subspectrum, whereas subtraction yields the S^α subspectrum. The pulse sequence based on Eq. [1] is outlined in Fig. 1a, and a four-step phase cycle can be found in Table 1. We dub it spin-state-selective excitation (S^3E).

S^3E is not the first attempt to selectively excite only one line of a doublet; another sequence can be found in Ref. (*14*), and more recent alternative approaches use heteronuclear coherence transfer echoes and pulsed field gradients (*15, 16*). The latter methods are, however, less sensitive than S^3E because the gradients prevent both subspectra being constructed from the same data set.

A first test of S^3E was to record 1D spectra in order to determine experimentally the degree of discrimination between the two doublet lines. For this purpose the pulse sequence in Fig. 1b was used; it has a filtering element preceding S^3E which suppresses I-spin magnetization from molecules without the hetero label S—a necessity for work

TABLE 1
Phase Cycles for Editing α or β S^3E Spectra[a]

				Receiver	
φ_1	φ_2	φ_3	φ_4	S^3E β	S^3E α
$\pi/4$	0	0	0	0	0
$\pi/4$	0	$\pi/2$	$\pi/2$	π	π
$\pi/4$	0	0	π	0	π
$5\pi/4$	0	$\pi/2$	$3\pi/2$	π	0

[a] In practice, for the full phase cycle initially two data sets are combined with the receiver phase of S^3E β: A (steps 1 and 2) and B (steps 3 and 4). Then $A + B$ and $A - B$ yield the two edited subspectra having a $\pi/2$ relative phase shift.

with ^{13}C or ^{15}N at the natural abundance level. Figure 2 illustrates the results of applying the basic two- and the four-step phase cycle in the S^3E element to a sample of ^{15}N-labeled RAP 17-97. Clearly, even the two-step cycle provides good discrimination between the two doublet components but small dispersive peaks are visible (marked with asterisks). These residual peaks are well suppressed using the four-step phase cycle which is the one recommended for practical use.

S-spin decoupling in t_1 is very useful in combination with S^3E E.COSY-type experiments because pairs of 1D sections for measurement of coupling constants are exactly the same in both subspectra and centered at the chemical shifts of the amide protons. In contrast, the corresponding sections in a normal E.COSY-type experiment are separated by a variable distance determined by a large J coupling constant. This is illustrated in Fig. 3.

Two-dimensional correlation experiments combining all the above features and extending earlier work of Willker and Leibfritz (*17*) employing a heteronuclear zero- (ZQ) or double-quantum (2Q) π rotation (*17–20*) after the homonuclear coherence transfer are outlined in Figs. 1c and 1d. These sequences can be used for measurement of $^1H-^1H$ J coupling constants between any pair of protons provided the homonuclear coherence transfer process can correlate them. For illustration with ^{15}N-RAP 17-97 the NOESY mixing variant was chosen, and it was found useful to employ the initial filtering element in order to eliminate the native magnetization of the aliphatic spectral region.

In Fig. 4 are shown H^N-H^α and tryptophan side chain cross peaks from the spectrum of ^{15}N-RAP 17-97 recorded with the sequence in Fig. 1c. As illustrated the editing according to the ^{15}N spin state makes it possible to measure the $^1H-^1H$ coupling constants. The typically small values of $^3J(H^N-H^\alpha)$ are in accordance with the predominantly α-helical structure of RAP 17-97 (*7, 8*).

Another possibility for gaining the same information about homonuclear coupling constants is by the pulse sequence in Fig. 1d. This is a heteronuclear 2D correlation

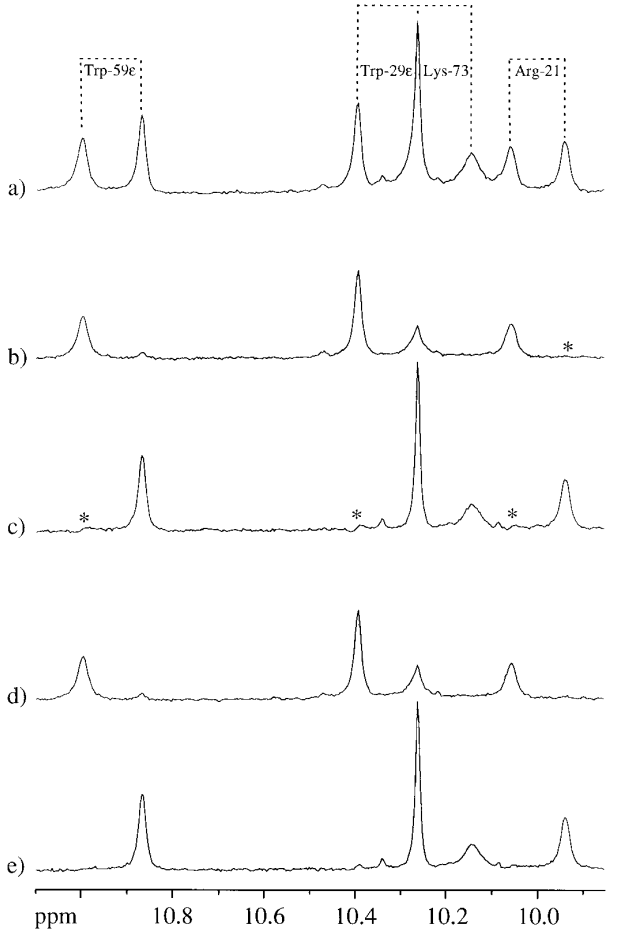

FIG. 2. ^{15}N-RAP 17-97 750-MHz Varian Unity Inova ^1H NMR spectra (part of amide region) (2.3 mM in 90%/10% H_2O/D_2O, pH 6.4, 20 mM NaCl, 1 mM phosphate buffer) recorded at 298 K with water presaturation and all representing 64 scans. The spectra represent (a) $\pi/2$ pulse excitation, (b) and (c) the S^3E sequence in Fig. 1b employing a delay of τ = 5.26 ms with the first and third entries, and (d, e) all four steps of the phase cycles in Table 1. Asterisks indicate residual dispersive cross talk between the edited subspectra.

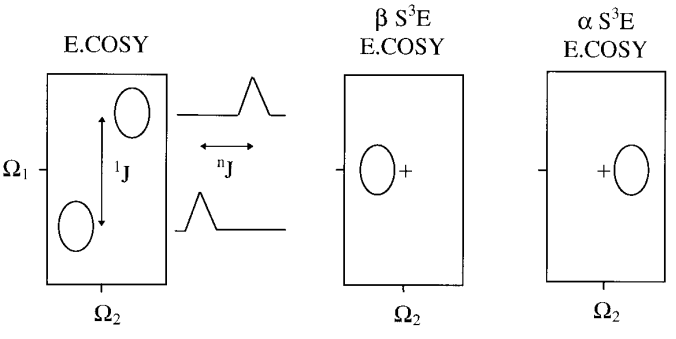

FIG. 3. Schematic spectra showing the differences between a normal E.COSY-type spectrum and edited S^3E E.COSY-type spectra with S decoupling in the t_1 period.

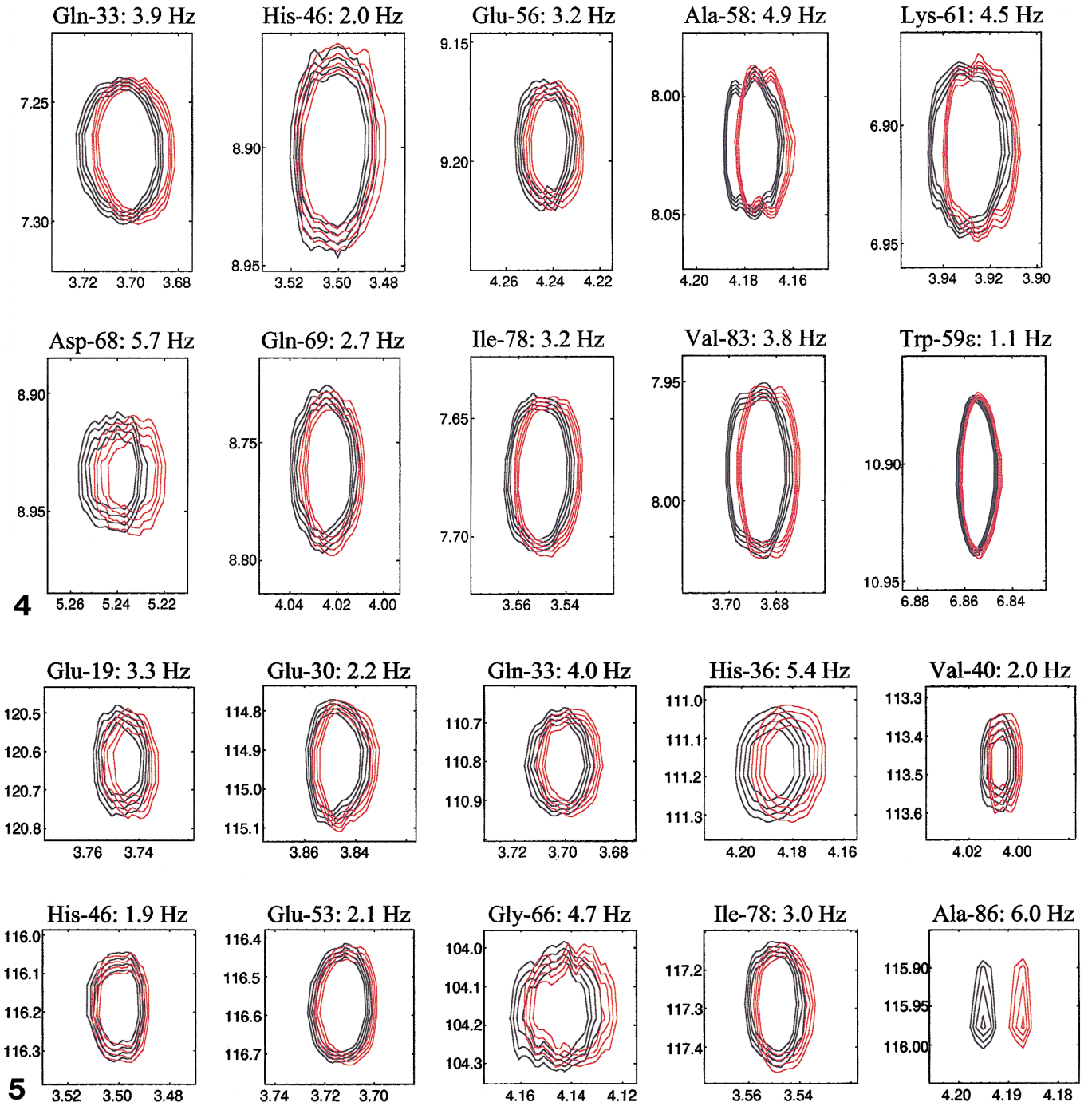

FIG. 4. Representative cross peaks from edited homonuclear S^3E J_{HH} NOESY spectra of ^{15}N-RAP 17-97 recorded with the sequence in Fig. 1c on a Varian Unity Inova 750-MHz spectrometer. The two edited subspectra (black and red, respectively) have been overlaid using the software package PRONTO (*21*). Parameters: NOESY mixing time, 60 ms; water presaturation, 1.5 s plus during NOESY mixing; t_1(max) = 25.6 ms; 64 scans; τ = 5.26 ms; Waltz decoupling in t_2; States–TPPI mode and cosine window functions. Spectral widths of 10,000 Hz were covered by a data matrix of 512 × 8192 zero-filled to 1024 × 8192 prior to Fourier transformation. The coupling constants were measured from 1D sections with an estimated precision of about ±0.2 Hz limited by digital resolution. A much higher accuracy can be obtained by taking the entire 2D peakshapes into account.

FIG. 5. Representative cross peaks from edited heteronuclear S^3E J_{HH} NOESY spectra of ^{15}N-RAP 17-97 recorded with the sequence in Fig. 1d on a Varian Unity Inova 750-MHz spectrometer. Same parameters as described in the legend to Fig. 4 except for 128 scans; t_1(max) = 42.6 ms. Spectral widths of 3000 × 10,000 Hz were covered by a data matrix of 256 × 8192 zero-filled to 512 × 8192 prior to Fourier transformation.

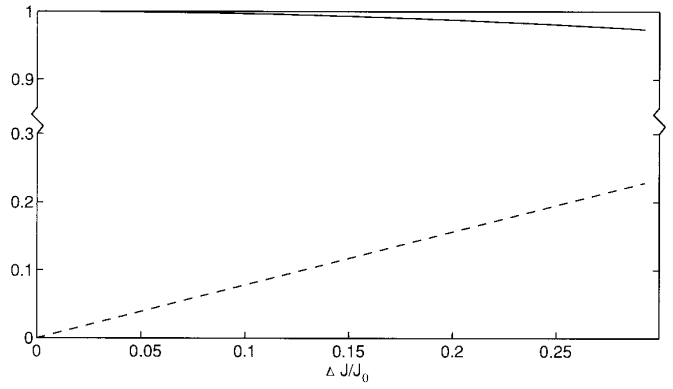

FIG. 6. Intensity functions for desired (full line curve) and undesired (dashed curve) doublet components in edited S^3E spectra as a function $\Delta J/J_0$ where $J = J_0 + \Delta J$ is the coupling constant of the actual doublet, and J_0 is that used in setting the delays of the S^3E element. The curves represent $|\cos(\pi J\tau) \pm \sin(\pi J\tau)|/\sqrt{2}$.

experiment where S^3E is employed on the S spin with respect to the I spin being in the α or β state, and a ZQ/2Q π rotation is used for magnetization transfer from S to I after t_1 and also after the mixing sequence. At the expense of a longer duration the sequence in Fig. 1d has advantages over that in Fig. 1c, namely the normally larger dispersion of S-spin chemical shifts, the absence of diagonal peaks, and the possibility of applying a heteronuclear gradient echo for artifact suppression. Figure 5 shows some results of applying this sequence to ^{15}N-RAP 17-97.

The relatively narrow range of $^1J_{NH}$ coupling constants in the backbone of proteins ensures excellent discrimination between the two doublet component in the edited S^3E subspectra. However, for larger ^1J ranges, cross talk will occur as illustrated quantitatively in Fig. 6. For $|\Delta J/J_0| = 12.7\%$ ($\tau = (2J_0)^{-1}$) the intensity ratio of the undesired to the desired component in the edited subspectra is 10%.

We should note that S^3E in no way changes the fact that E.COSY-type and also other pulse sequences are susceptible to the relaxation effects described in Refs. (*22–26*). A consequence is that although separation of resonances can be measured precisely these separations need to be corrected for relaxation effects in order to determine the J coupling constants. When pertinent submultiplets (e.g., corresponding to α or β spin states of ^{15}N) are well separated in a multidimensional E.COSY-type spectrum the accuracy of the measurement of resonance separations is mainly determined by the signal-to-noise ratio.

In conclusion, we have introduced and demonstrated a novel heteronuclear nonselective pulse sequence element, S^3E, that in doublets selectively excites resonances corresponding to exclusively either the α or the β spin state of the coupled heteronuclear spin. This selectivity forms the basis for an E.COSY-type extraction of J coupling constants; however, instead of having one spectrum with two peaks (corresponding to the α or β spin state of the coupled spin), the new method generates two spectra each with only one peak for each spin system. Since these two spectra are generated from the same data set, there is no reduction in sensitivity compared to conventional methods. On this basis we expect S^3E to be a valuable addition to a wide range of E.COSY-type experiments where one-bond coupling constants serve to make possible the measurement of smaller coupling constants. Finally, S^3E will also be very useful in connection with automatic extraction of coupling constants by computer because there is no variable $^1J_{IS}$ separation of pertinent 1D sections when S-spin decoupling is applied during the evolution period.

ACKNOWLEDGMENTS

The spectra presented have been recorded on the 750-MHz Varian Unity Inova spectrometer of the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules at Carlsberg Laboratory. We thank Peter R. Nielsen, Lars Ellgaard, Michael Etzerodt, Hans C. Thøgersen, and Flemming M. Poulsen for the loan of the ^{15}N-RAP 17-97 sample and for the assignment. A.M. is supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

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