

15 March 1996

Chemical Physics Letters 251 (1996) 102-110



Double quantum two-dimensional Fourier transform electron spin resonance: distance measurements

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Received 4 December 1995; in final form 8 January 1996

Abstract

The first double quantum two-dimensional Fourier transform electron spin resonance (2D-FT ESR) experiments are reported. Extension of 2D-FT ESR to enable detection of the $\Delta M_s = \pm 2$ transition enhances the capability of ESR in studying structural properties (i.e. distances in bilabeled molecules). A distance of 21 Å was found for a poly-proline peptide, spin labeled at both ends, in agreement with earlier measurements using fluorescence energy transfer.

1. Introduction

Double quantum NMR has proved to be a valuable tool for characterizing structural properties in a variety of samples ranging from isotropic solutions to single crystals and including oriented liquid crystalline solutions as well as polycrystalline powders. The various applications include simplification of complicated single quantum spectra [1,2], determination of spin connectivity and topology [3,4], obtaining high resolution spectra in inhomogeneous magnetic fields [5] and facilitation of coherence transfer and indirect detection in systems of magnetically rare nuclei [6].

In ESR the observation of electron spin transitions other than $\Delta M_s = \pm 1$ has been restricted to the cw ESR detection of the 'half-field' transition [7]. Recent developments in two-dimensional Fourier transform (2D FT) ESR methodology have encouraged us to adapt the well known NMR techniques to the ESR case. A particularly valuable application for ESR would be the measurement of distances ≥ 20 Å in double labeled macromolecules or between paramagnetic sites in a solid.

Distance measurements in ESR thus far largely rely on a large exchange or dipolar coupling leading to observable differences in the continuous wave spectrum [8,9]. Hence they have been restricted to measurements of less than ≈ 15 Å. However, selective pulse methods have been developed based upon electronelectron double resonance [10,11] and upon the '2+1'-pulse train [12,13] using electron spin echoes. The advantage of double quantum ESR would be that one can, with appropriate phase cycling, isolate just the double quantum coherence signal from a double labeled molecule from which the dipolar interaction is directly extracted from the observed modulation pattern.

The use of double spin labeling of macromolecules for distance measurements is analogous to the method of fluorescence energy transfer (FET). However, distances measured by FET suffer from large uncertainties in the κ^2 parameter that describes the relative orientation of the transition dipoles of the two chromophores [14]. No comparable problem exists in



Fig. 1. Pulse sequences used to generate the double quantum coherence. (a) The basic three-pulse sequence and (b) a five-pulse sequence obtained by including π pulses during the preparation and evolution periods.

ESR, since the spins are quantized along the magnetic field.

We report below on our successful observation of the two-quantum coherence signal and show how it is easily utilized for distance measurements.

2. Experimental

All experiments were performed on the 2D FT ESR spectrometer described elsewhere [15–17]. Additional modifications included the ability to perform five- and six-pulse sequences with full phase cycling. The use of a TWTA with an output power of 2 kW and our specially designed loop gap resonator allowed uniform coverage over a spectral range of about ± 100 MHz.

All experiments reported here were performed between -83 to -85° C. At this temperature the T_2^* (≈ 8 ns) were too short to allow the detection of the free induction decay, but an echo decay can readily be measured. In all cases the signal was collected after the instrumental dead time, and the full echo was retained.

Fig. 1 shows the three- and five-pulse sequences generally used to detect double quantum coherences. We used the five-pulse sequence for our experiments, because it contains π pulses during the preparation and evolution periods which refocus all the coherences, thereby eliminating the large dephasing effects from T_2^* (without affecting the dipolar interaction, cf. Section 4). Such a technique is commonly used in NMR

Table 1

Phase cycling used for the five-pulse sequence. This four step cycle is augmented by the additional four steps in parentheses, to which CYCLOPS is then added

$\pi/2$	π	$\pi/2$	π	$\pi/2$	Sign
0 (180)	0 (0)) 0 (0)	0 (0)	0 (0)	+ (-)
90 (270)	90 (90) 90 (90)	0 (0)	0 (0)	- (+)
180 (0)	180 (180) 180 (180)	0 (0)	0 (0)	+ (-)
270 (90)	270 (270) 270 (270)	0 (0)	0 (0)	- (+)

[18], and we found it essential in the present experiments because of the shortness of T_2^* . (We could not obtain any signal by the three-pulse sequence when using the two-quantum coherence phase cycling (cf. below) in accordance with these matters.) Two classes of experiments were performed on each sample. Initially the duration t_p was varied at a fixed t_1 . The value of t_1 was typically fixed at the minimum achievable (usually 50 ns). This experiment will be referred to as the double quantum modulation experiment (DQM). In the second experiment the duration t_1 was varied. The value of t_p was kept constant at the optimum determined from the DQM experiment. This experiment will be referred to as double quantum COSY (DQ-COSY).

For the DQM experiment the initial value of t_p was 50 ns and 128 steps in t_p at 1 ns step size were collected. For each t_p step, 256 data points at a 1 ns interval were measured in t_2 after a spectrometer dead time of 40 ns.

From the DQM experiment the optimum value of t_p was determined as 90 ns. In the DQ-COSY experiment 128 points in t_1 at 1 ns step size were used after a t_1 dead time of 50 ns.

We show all spectra in magnitude format. The frequency domain DQ-COSY spectrum was obtained by Fourier transforming with respect to $2t_1$. Also a filter function was used along both $2t_1$ and t_2 to eliminate clipping artifacts in the complex transform.

For both experiments a 32 step single quadrature phase cycling scheme was used to cancel signals arising from undesired coherence transfer pathways (i.e. zero and single as well as higher quantum coherences). The phase cycling was based on the four step cycle proposed by Braunschweiler et al. and Bodenhausen et al. [4,18] (cf. Table 1). An additional four step cycle was added, by advancing the phase of the first pulse by 180° and subtracting the signal (cf. entries in parentheses in Table 1). This had the advantage of suppressing background signals for small values of t_2 and also of canceling some extra coherence pathways. Inclusion of CYCLOPS [15,16] made this into a 32 step cycle. The nominal width of a $\pi/2$ pulse was 4.9 ns while a π pulse was about 9.6 ns.

The synthetic peptides we studied were (a) A-P-P-P-P-C-MTSSL (I) (monoradical) and (b) MTSSL-C-P-P-P-C-MTSSL (II) (biradical) where (i) MTSSL is the (1-oxyl-2,2,5,5-tetramethyl-pyrroline-3-methyl) methanethiosulphonate spin label and (ii) A, C and P are amino acids alanine, cysteine and proline, respectively. These short chain peptides were custom synthesized and labeled with the MTSSL spin label, via a disulphide bond [20,21], at the Cornell Biotechnology Laboratory. The MTSSL spin label was obtained from Reanal (Budapest, Hungary). The concentrations of the two systems were about 1.5 mM. The sample was prepared by dissolving the required amount of the peptide in a glycerol/water/triflouroethanol mixture which was buffered with MOPS (3-[N-morphilino] propane sulphonic acid).

The system of poly-proline has not only been rigorously characterized [22] but was also traditionally used to determine spin distances using FET [23]. Besides they are relatively easy to prepare. Stryer and Haughland [23] determined the distance of this 4-mer to be about 20 Å.

3. Results

We first show in Fig. 2 the standard SECSY-ESR spectra from the pulse sequence $\pi/2 \xrightarrow{t_1} \pi/2 \xrightarrow{t_1+t_2} [15-17,19]$ obtained from the bilabeled (Fig. 2a) and monolabeled (Fig. 2b) peptides at -85° C. (The frozen ESR spectrum appears along the f_2 axis, and the homogeneous widths are displayed along the f_1 axis. The actual lineshapes along f_2 are, of course, those of the ESR spectra in the magnitude mode and they are modified in their appearance by (a) the finite 'deadtimes' after both the first and second pulses and (b) the fall-off of intensities in the wings due to imperfect coverage (cf. Section 4)). Figs. 2a and 2b are nearly identical in appearance, since the electron-spin dipolar interaction in the biradical is a small perturbation, the dominant interaction terms being the hyperfine (hf) interaction of each nitroxide electron spin with the respective ¹⁴N nucleus, as well as its g-tensor interaction. They are the source of the observed $T_2^* \sim$ 8 ns. in these spectra. A careful examination does show that the homogeneous T_2 is a little shorter in the biradical (246 ns) compared to that of the monoradical (289 ns) for this frozen sample reflecting the effects of the weak dipolar interaction. The temperature of -83°C was selected for the double quantum study, because it represents a maximum in T_2 for these labeled peptides.

In Fig. 3 we show the time-domain result of a typical DQM experiment on the biradical in which t_p is stepped out. Note in the contour plot (Fig. 3b) that there are echoes at $t_2 = t_p$ and $t_2 = t_p + 50$ (i.e. $t_2 = t_p + t_1$, (as well as other weaker echoes at $t_2 = 2t_p$ and $t_2 = 2t_p + t_1$). The strongest echo occurs at $t_2 = t_p$. We show in Fig. 3a the magnitude signal versus t_p and t_2 . For simplicity in viewing we display only the echo signal that occurs for $t_2 = t_p$, and we show it along the t_p axis, so that the t_2 axis is almost orthogonal to the viewing direction. Note its oscillatory behavior. By contrast, the equivalent experiment performed on the monoradical (not shown) gives no signal. It is therefore clear that the DQM signals in Fig. 3 arise as a result of the dipolar interaction in the biradical, and it must arise from double quantum coherence since the phase cycling filters out the other coherences. The maxima appear at $t_p = 90$ and 150 ns. Assuming a fixed distance between the nitroxide labels, the development of the double quantum coherence is directly proportional to $\sin^2(\bar{a}t_p/2)$ where $\bar{a} =$ $3\gamma_e^2\hbar/r^3$ is the dipolar interaction, as we show in the next section. This corresponds to a spin-spin distance of 21 Å, which is in good agreement with the FET measurement of 20 Å [23].

We show in Fig. 4 the DQ-COSY obtained from the echo which forms at $t_2 = t_p$. The single-quantum ESR spectrum appears along the f_2 axis, while the double quantum ESR spectrum is along the f_1 axis. The single quantum ESR spectrum is similar to, but not identical to that observed in the SECSY-ESR of Fig. 2. The double quantum spectrum has, of course, been largely refocussed by the second π pulse of the five-pulse sequence.



Fig. 2. A comparison of the magnitude SECSY-ESR spectra obtained from the poly-proline peptide (a) doubly spin-labeled and (b) singly spin labeled at -85° C.

4. Discussion

We wish to discuss how the observed two-quantum signal arises, and why it permits such a simple analysis

for the distance measurement. To do this, we first adapt the conventional theory for double-quantum coherence in NMR [24,25] to the present ESR case. For this purpose we consider a simplified form [24] of the



Fig. 3. A typical 2D double quantum modulation (DQM) spectrum, obtained by varying the preparation period and measuring the signal versus t_2 . The magnitude time domain signal is shown in both (a) stack and (b) contour formats. (a) only shows the echo for $t_2 = t_p$.



Fig. 4. The frequency domain DQ-COSY spectrum. The f_1 axis was obtained by Fourier transforming with respect to $2t_1$. A filter function was used in both dimensions. The magnitude spectrum is shown in both (a) stack and (b) contour formats for the echo at $t_2 = t_p$.

biradical spin-Hamiltonian:

$$H = \hbar \Omega_1 S_{1z} + \hbar \Omega_2 S_{2z} + \hbar b (3S_{1z} S_{2z} - S_1 \cdot S_2), \quad (1)$$

where

$$b = \gamma_{\rm e}^2 \hbar (1 - 3\cos^2\theta) / 2r^3.$$
 (2)

Here Ω_1 and Ω_2 represent the resonance offsets for the electron spins 1 and 2 on the two different nitroxide labels. The dependence of these resonance offsets on the hf and g tensors and their orientations are only implicitly included in this simplified discussion. The term in b in Eq. (1) is the secular part of the dipolar interaction between the electron spins. It is easy, using standard methods [24], to follow the spin evolution for the relevant coherence pathways associated with the pulse sequences in Fig. 1.

We first consider the double quantum coherence which would be predicted for the sequence of three non-selective $\pi/2$ pulses in Fig. 1. The first $\pi/2$ pulse creates single quantum coherences, which are modified by the dipolar interaction during the preparation period t_p , so that they are converted into double quantum coherences by the second $\pi/2$ pulse. After the evolution period, t_1 , a third $\pi/2$ pulse transfers the double quantum terms back into single quantum coherences, which can then be detected during t_2 . This development of the coherences can most readily be followed by expansion of the density matrix in terms of irreducible spherical tensor operators (ISTO) [25-27], T_{LM} representing the *M*th component of rank L for two coupled electron spins. The sequence then becomes $T_{10} \xrightarrow{\pi/2} T_{1\pm 1} \xrightarrow{t_p} T_{2\pm 1} \xrightarrow{\pi/2} T_{2\pm 2} \xrightarrow{t_1} T_{2\pm 2} \xrightarrow{\pi/2}$ $T_{2\pm 1} \xrightarrow{t_2} T_{1\pm 1}$. In the five-pulse experiment in Fig. 1, the first π pulse refocusses $T_{2\pm 1} \rightarrow T_{2\mp 1}$ during the preparation period and the second π pulse refocusses $T_{2\pm 2} \rightarrow T_{2\mp 2}$ during the evolution period. A Hamiltonian of the form of Eq. (1) allows an analysis of double quantum coherence by well-known density matrix methods, (i.e. the product operator method [24,25]). The three-pulse case of Fig. 1 is treated in detail by this method in ch. 9 of Slichter's monograph [24]. It is straightforward to extend this method to the fivepulse sequence in Fig. 1 for the Hamiltonian of Eq. (1) to obtain the signal $\langle F^+ \rangle$:

$$\langle F^+(t_p, t_1, t_2) \rangle_5^p \propto \sin(at_p) \sin(at_2/2) \times [\exp\{-(i\Omega_1 t_2)\} + \exp\{-(i\Omega_2 t_2)\}]/2, \qquad (3)$$

where $a = a(\theta) \equiv 3b$. The superscript p implies the primary two-quantum signal, and the subscript refers to the number of pulses. Because of the deadtime t_d in t_2 this signal would not be observable, as a result of the inhomogeneous spread in Ω_1 and Ω_2 . However, by adding a π pulse at time t_2 , one expects to form the echo after this pulse. The signal becomes (by the same method)

$$\langle F^+(t_p, t_1, t_2, \tau) \rangle_6^p \propto -\sin(at_p) \sin(at_2/2) \cos\{a(t_2 + \tau)/2\} \times [\exp\{-(i\Omega_1\tau)\} + \exp\{-(i\Omega_2\tau)\}]/2,$$
(4)

where $\tau \equiv t_3 - t_2$ with t_3 the time after the sixth pulse. Eq. (4) predicts an echo at $\tau = 0$. We have tried to observe this primary echo signal, but without success (cf. below).

What then is the source of the echoes shown in Fig. 3? We note that our pulses are not truly non-selective. We estimate $B_1 \approx 18$ G in the rotating frame, whereas the extent of the nitroxide spectrum in Fig. 2 is about ± 42 G (the good spectral coverage we experience is for magnitude spectra, where rotation just into the xy plane by a $\pi/2$ pulse is sufficient [15,16,28]). Two types of imperfections are relevant for us: (1) the rotation of the spins is not precisely π or $\pi/2$ across the spectrum; (2) the dipolar term $a/\gamma_e = 3b/\gamma_e$ in our experiment is about 2.3 G, so it can act as a perturbation during the pulse, thereby producing extra coherences from the pulse [29,30]. We have examined all possible coherences (the T_{LM}) arising from the five-pulse sequence that can survive the phase cycling. We find only one coherence pathway that is consistent with our observations on the echo that forms at $t_2 = t_p$, (with other distinct pathways appropriate for the other 'forbidden' echoes¹).

¹ The other echoes are consistent with the pathways (a) $T_{10} \xrightarrow{\pi/2} T_{1\pm 1} \xrightarrow{l_p} T_{2\pm 1} \xrightarrow{\phi} T_{20} \xrightarrow{l_p'} T_{20} \xrightarrow{\pi/2} T_{2\pm 2} \xrightarrow{l_1} T_{2\pm 2} \xrightarrow{\beta} T_{2\mp 1} \xrightarrow{l_1} T_{2\mp 1} \xrightarrow{\pi/2} T_{2\mp 1} \xrightarrow{\pi/2} T_{2\mp 1} \xrightarrow{t_1} T_{2\mp 1} \xrightarrow{\tau/2} T_{2\pm 1} \xrightarrow{t_2} T_{1\pm 1}$ for the echo at $t_2 = t_p + t_1$; (b) $T_{10} \xrightarrow{\pi/2} T_{1\pm 1} \xrightarrow{t_p} T_{2\pm 1} \xrightarrow{\phi} T_{2\pm 1} \xrightarrow{\tau/2} T_{2\pm 1} \xrightarrow{\pi/2} T_{2\pm 1} \xrightarrow{\pi/2} T_{2\pm 1} \xrightarrow{\pi/2} T_{2\pm 1} \xrightarrow{\pi/2} T_{2\pm 2} \xrightarrow{\mu} T_{2\mp 2} \xrightarrow{\pi/2} T_{2\mp 2} \xrightarrow{\pi/2} T_{2\mp 1}$ if or the echo at $t_2 = 2t_p$ and (c) $T_{10} \xrightarrow{\pi/2} T_{2\mp 2} \xrightarrow{\pi/2} T_{2\mp 1} \xrightarrow{t_1} T_{1\pm 1}$ for the echo at $t_2 = 2t_p$ and (c) $T_{10} \xrightarrow{\pi/2} T_{2\pm 2} \xrightarrow{\pi/2} T_{2\pm 1} \xrightarrow{\pi/2} T$

We summarize it with the ISTO $T_{10} \xrightarrow{\pi/2} T_{1\pm 1} \xrightarrow{t_p} T_{2\pm 1} \xrightarrow{\phi} T_{2\pm 1} \xrightarrow{\phi} T_{20} \xrightarrow{\pi/2} T_{2\pm 2} \xrightarrow{t_1} T_{2\pm 2} \xrightarrow{\pi} T_{2\pm 2} \xrightarrow{t_1} T_{2\pm 2} \xrightarrow{\pi} T_{2\pm$

$$\langle F^{+}(t_{p},\theta,t_{p'},t_{1},t_{2})_{5}^{f} \\ \propto [a/(4\gamma_{e}B_{1})]\sin(at_{2}/2)\sin(at_{p}/2)\sin(2\phi) \\ \times [\exp(-i\Omega_{1}t_{2}) + \exp(-i\Omega_{2}t_{2})] \\ \times [\sin(\Omega_{1}t_{p}) + \sin(\Omega_{2}t_{p})],$$
 (5)

where the superscript f implies a forbidden signal. This signal is seen to form an echo at $t_2 = t_p$ with an oscillation given by $\sin^2(at_p/2)$. The factor $a/\gamma_e B_1$ arises from the perturbational mixing in of the forbidden coherence [29,30]. [We found it possible to double the power in our low Q bridged loop gap resonator, thereby increasing B_1 . This did have the effect of suppressing the 'forbidden' echoes]. Because Eq. (5) results from the retention of the forbidden signal after the second $\pi/2$ pulse it cannot reduce to Eq. (3) if we set $\phi = \pi$, since Eq. (3) involves the $\Delta M_s = \pm 1$, i.e. the allowed transition.

In the isotropic glass this signal has to be averaged over the orientations, θ , of the radial vector \mathbf{r} between the two spins to obtain the final signal:

$$F_j^k \propto \int_0^{\pi} \langle F^+ \rangle_j^k \sin \theta \, \mathrm{d}\theta \,. \tag{6}$$

In Fig. 5a we show the double quantum signal obtained from inserting Eq. (5) into Eq. (6) and setting $t_2 = t_p$ corresponding to the echo maximum. Also we have set r = 21 Å. The basic features (after a 50 ns deadtime) are seen to be very similar to the experimental observation of Fig. 3a. Furthermore, the oscillations have a simple periodicity consistent with setting $|1 - 3\cos^2 \theta|/2 \approx 1$ in Eq. (2) corresponding



Fig. 5. Simulated time domain double quantum signal obtained for (a) forbidden coherence pathway ($t_2 = t_p$) and (b) primary coherence pathway ($t_2 = 50$ ns and $\tau = 0$ ns). The effect of T_2 is included.

to $\theta \approx 0$. This is the result of the fact that the forbidden pathway depends upon $a/\gamma_e B_1$ which thereby produces an orientational selectivity.

When we calculate F_6^p by inserting Eq. (4) into Eq. (6) we obtain the result of Fig. 5b for $\tau = 0$ and $t_2 = 50$ ns. It is clear from this figure (as well as the predicted features for $t_p < 50$ ns, which are not shown), that the range of orientations of the dipolar interaction acts to smear out the frequency of the 'averaged' echo decay, because of the absence of any orientation selectivity. Thus the 'forbidden' coherence pathway is actually the more favorable for extracting distances. (Factors which would be expected to lead to additional smearing out are a distribution in the values of r and any small effects from the intermolecular dipolar interaction.)

Since the poly-proline peptides are fairly rigid, we may expect that the orientational selectivity of r will imply some orientational selectivity in the magnetic

 $[\]begin{array}{cccc} T_{1\pm 1} & \stackrel{\prime p}{\longrightarrow} T_{2\pm 1} & \stackrel{\phi}{\longrightarrow} T_{2\pm 1} & \stackrel{\prime p'}{\longrightarrow} T_{2\pm 1} & \stackrel{\pi/2}{\longrightarrow} T_{2\pm 2} & \stackrel{\prime 1}{\longrightarrow} T_{2\pm 2} & \stackrel{\beta}{\longrightarrow} T_{2\mp 1} & \stackrel{\prime 1}{\longrightarrow} \\ T_{2\mp 1} & \stackrel{\pi/2}{\longrightarrow} T_{2\pm 1} & \stackrel{\prime 2}{\longrightarrow} T_{1\pm 1} & \text{for the echo at } t_2 = 2t_p + t_1. \text{ Note that the second π pulse is replaced by a pulse of flip angle β.} \end{array}$

tensors of the nitroxide. This might account, in part, for the differences in the spectrum along f_2 in Fig. 4a from that in Fig. 2a. Furthermore, the lack of selectivity in the F_6^p signal may (in conjunction with the finite and imperfect pulses) result in inadequate refocussing by the π pulses, of the broad spectral distributions, especially of the two quantum coherence during the evolution period. This could be a partial explanation for the inability to observe this signal.

Finally, we note that if in the $F_5^{\rm F}$ signal there is adequate refocussing, then the spectrum along f_1 in Fig. 4 should be reasonably approximated as a Lorentzian of width $(T_2^{\rm DQ})^{-1}$. Simple considerations [24,25,31] show that for secular (and pseudo-secular) contributions to T_2 from the magnetic tensors of the nitroxides, and for the dipolar interaction playing only a minor role, then $T_2^{\rm DQ} \approx T_2^{\rm SQ}/2$ (where SQ = single quantum) if the dynamic processes modulating the two nitroxides are uncorrelated, whereas $T_2^{\rm DQ} \approx T_2^{\rm SQ}/4$ if they are fully correlated. A comparison of Fig. 4 with Fig. 2a leads to a $T_2^{\rm DQ} \sim T_2^{\rm SQ}/4$. While it would be premature to interpret this preliminary result as a significant argument for correlated dynamics (e.g. overall rotation as opposed to local motions), it illustrates the potential value of such comparisons.

5. Conclusions

Two-dimensional Fourier transform ESR has been extended to obtain double quantum transitions. These experiments demonstrate the potential of ESR as a valuable tool for elucidating structural properties². It was shown that distances in a bilabeled molecule can be accurately read-off from the 2D DQM spectrum. We expect this method will enable measurements of distances as great as 50–60 Å. Careful analysis of the double quantum signal could also provide information on molecular dynamics in conjunction with single quantum spectra.

Acknowledgement

We wish to thank Dr. D.E. Budil and R.H. Dr. Crepeau with their help in the initial stages of this work and Dr. D. Zax for a helpful discussion. This work was supported by NIH Grant RR07126 and NSF Grant CHE 9313167.

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 $^{^{2}}$ A referee has called our attention to the work of S.A. Dzuba, M.K. Bosch, and A.J. Hoff, Chem. Phys. Letters (in press), in which a double quantum coherence was observed by ESE from a photo-induced radical-pair, using a two-pulse sequence after the laser pulse as originally suggested by Tang and Norris [32]. This system has a smaller spectral bandwidth than the nitroxide system.

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