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2D-REFINE spectroscopy: Separation of overlapping hyperfine spectra

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Abstract

We show on a mixture of three spectrally overlapping paramagnetic compounds TEMPO, BDPA and CuHis that it is possible to separate their field-swept and hyperfine spectra based on the difference in their longitudinal relaxation times T_1. This was achieved in a two-dimensional experiment, where one dimension corresponds to the spectral domain and the second dimension encodes the relaxation behavior of the individual compound. Inverse Laplace Transform with respect to this domain separates the field-swept and hyperfine spectra of the individual compounds in the relaxation rate domain. This extends our formerly proposed Relaxation Filtered Hyperfine (REFINE) method to be applicable to more than two spectrally overlapping spectra by adding a further dimension to the chosen EPR experiment.

Keywords: REFINE; Hyperfine spectroscopy; EPR; Relaxation; Paramagnetic centers; Spectral separation

1. Introduction

One- and two-dimensional hyperfine spectroscopy such as ENDOR (Electron Nuclear Double Resonance), ESEEM (Electron Spin Echo Envelope Modulation) or HYSCORE (Hyperfine Sublevel Correlation) spectroscopy, is a powerful tool to investigate the local environment of paramagnetic centers in a distance range up to 0.8 nm with high precision. Whereas the isotropic hyperfine coupling reveals details of the electronic structure of the unpaired electron, precise structural information of the nuclei can be obtained by the dipolar contribution [1,2]. However, in the presence of more than one paramagnetic species, these methods suffer from an intrinsic low resolution due to the overlap of the individual hyperfine spectra. In many studies of biological systems for example, more than one paramagnetic species contributes to the observed hyperfine spectra at a specific field value. This severely hinders the assignment and quantitative interpretation of such spectra. In this case hyperfine spectra can be taken at different field positions within the EPR spectra, experiments can be performed at different microwave frequencies and, if possible, biochemical mutations of the biological system can be used to assign the hyperfine spectra to the individual paramagnetic species [3].

Due to an increased sensitivity and a better spectral resolution for low-γ nuclei, ENDOR experiments performed at high magnetic fields (95 GHz and higher) can dramatically improve and simplify hyperfine spectra [4–7]. Furthermore, at high magnetic fields it is often possible to resolve all components of the anisotropic g-tensor. This orientation selectivity allows the determination of the orientation of the hyperfine tensor with respect to the g-tensor axis system even in disordered powder samples [8–10]. Unfortunately this advantage does not hold for methods like ESEEM or HYSCORE, since they rely on forbidden transitions whose transition moments are considerably attenuated at high magnetic fields [11]. Additionally, all pulsed hyperfine experiments are performed under solid state conditions leading to a broad powder spectra, dominated by anisotropic hyperfine or g-tensor interactions. Therefore,

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it is generally not possible to separate spectrally overlapping paramagnetic species by performing experiments at higher magnetic fields.

Pulsed hyperfine spectroscopy experiments are usually performed at low temperatures, because of fast electron spin relaxation. Since different paramagnetic species (e.g. organic radicals, metal-centers or clusters) show big differences in relaxation times, especially at low temperatures, this difference can be used to separate their individual contributions with respect to the overall detected signal. Recently we demonstrated this concept and developed REFINE spectroscopy, based on an inversion-recovery relaxation filter to suppress one compound out of a mixture of several species. In the presence of only two spectrally overlapping species this method can be applied to record one- and two-dimensional hyperfine spectra of the individual compounds [12]. We successfully applied this method to separate the hyperfine spectra (ESEEM) of the two iron–sulfur (FeS) clusters N1 and N2 in Complex I of the mitochondrial respiration chain at a temperature of 16 K [13].

Here we show that REFINE spectroscopy is capable to separate more than two spectrally overlapping species. For this, the experiment is extended into a further dimension, which encodes the relaxation behavior of the individual species. If the species under study show a difference in their relaxation behavior, an inverse Laplace transformation along this dimension leads to a separation of the different signals. Since the numerical inverse Laplace is highly unstable, we used a robust fitting algorithm, similar to DOSY spectroscopy in NMR [14], to perform this task. We demonstrate the success of the method on a mixture of three model compounds and the separated EPR and hyperfine spectra are compared on a qualitative basis.

2. Materials and methods

2.1. Sample preparation

A mixture of three organic radicals BDPA (α,β-bisphenylene-β-phenylallyl-benzolate) and TEMPO (2,2,6,6-tetramethyl-piperidine-1-oxyl) diluted in polystyrene, and a perdeuterated copper–histidine complex (CuHis) were chosen as model system.

BDPA and TEMPO are first separately dissolved in a solution of polystyrene in chloroform. The solvent is then slowly evaporated at 30 °C and the polystyrene film finally dried under high vacuum (10^{-5} mbar) to ensure that all solvent has been removed. The final concentrations (w/w) of the radical in the polystyrene matrix are 2% BDPA and 0.1% TEMPO, respectively.

The CuHis complex was prepared by mixing 0.5 ml of a 0.1 M $CuSO_4$ aqueous solution and 50 ml of a 0.1 M $ZnSO_4$ aqueous solution with 50 ml of a 0.2 M aqueous solution of L-hystidine hydrochloride. All solvents are deuterated. Polycrystalline material was obtained from by slow evaporation of the water over a period of a few days and the crystals were crushed prior mixing with the other two radicals.

In the mixture, the final concentrations of the three species TEMPO, BDPA and CuHis were chosen to be 5×10^{-3} mol/Kg, 2×10^{-3} mol/Kg and 2×10^{-2} mol/Kg, respectively, such that the maximum echo intensities of the three EPR signals were on the same order of magnitude. In the REFINE experiment, the CuHis concentration from the mixture was increased to 1×10^{-1} mol/Kg. The sample was not degassed, leading to faster relaxation rates due to the presence of oxygen.

The experiments were performed on a Bruker E-580 X-band spectrometer using a Bruker dielectric EPR cavity (MD5-W1) equipped with an Oxford helium flow cryostat (CF935) at a temperature of 20 K. Microwave pulses were amplified using a 1 kW pulse TWT amplifier (Applied Systems Engineering). Field swept spectra were obtained by recording the integrated Hahn-echo ($\pi/2$–τ–π) intensity as a function of the external magnetic field. The pulse lengths used in the experiments are 12 ns for the $\pi/2$ and 24 ns for the π pulses, respectively. Initial saturation of the polarization was achieved by using a picket-fence (PF) pulse sequence of 28 successive π pulses with a pulse separation of 2 μs, prior detection. The PF sequence was optimized by observing the suppression of the Hahn-echo for short values of T. The time increment T in the relaxation encoded dimension was increased logarithmically ($T^{i+1} = T^i + i \cdot \Delta t$). To remove unwanted echoes and any offsets, a 4-step phase cycle was used (see Table 1) [15].

To obtain the hyperfine frequency domain in the two-dimensional REFINE–ESEEM spectra the time domain is high-pass filtered to remove the echo decay function and a Hanning window function is applied. Prior to Fourier transformation the data set is zero-filled to the double number of data points.

2.2. Data processing

The data set obtained in the 2D-REFINE experiment can be described by the following integral

$$S(\tau, T) = \int\int I(v, R) \cdot e^{iv2\tau} \cdot G(R, T) dv\, dR, \quad (1)$$

with $S(\tau, T)$ the two-dimensional experimental data set, R the relaxation rates and $G(R, T)$ the relaxation kernel describing the decay function in the relaxation encoded domain, and $I(v, R)$ the hyperfine spectra amplitudes. For a saturation recovery experiment the relaxation kernel is of the form

Table 1
Phase cycle sequence

Step	P1	P2	P3	Sum
1	$+y$	$+x$	$+x$	+
2	$+y$	$-x$	$+x$	−
3	$-y$	+x	$+x$	+
4	$-y$	$-x$	$+x$	−

$$G(R,T) = 1 - e^{-T\cdot R}, \quad (2)$$

Other relaxation kernels can be chosen (based on T_1 or T_2) such as an inversion-recovery ($\propto T_1$) or a Hahn-echo decay ($\propto T_2$) kernel. For broad EPR lines the saturation recovery experiment minimizes the effect of spectral diffusion and shows best performance as described above.

To obtain the desired two-dimensional hyperfine spectrum $I(v,R)$ from $S(\tau,T)$ Eq. (1) has to be inverted. This can be achieved by an inverse Fourier transformation (iFT) of S with respect to τ followed by an inverse Laplace transformation (iLT) with respect to T in order to obtain the relaxation rate R of each individual species

$$S(\tau,T) \overset{\text{iFT}(\tau)}{\longrightarrow} P(v,T) \overset{\text{iLT}(T)}{\longrightarrow} I(v,R) \quad (3)$$

In contrast to the inverse Fourier transformation, computing the inverse Laplace transformation of an experimental data set is an ill-conditioned problem and one that may be intractable [16]. Numerous strategies have been proposed to implement a robust numerical inversion of exponential recovery data in terms of their rate components. Here a least-square fitting routine is used similar to the one described in [17].

After Fourier transformation of the experimental data with respect to τ the data set $P(v,T)$ is obtained, which can be approximated by a finite sum of discrete relaxation rates R_i of the form:

$$P(v,T) = \sum_i A^i \cdot (1 - e^{-T\cdot R^i}), \quad (4)$$

with $A_i(v)$ describing the amplitudes that each particular rate constant R_i has to the hyperfine frequency v. These amplitudes are determined by a non-negative least-squares fitting routine to minimize σ for each frequency v.

The amplitude vectors $A_i(v)$ obtained for each particular frequency v given by the inverse Fourier-transform assemble to the complete two-dimensional spectrum $I(v,R)$. In the case of EPR spectra, where the data set consists initially of a set $S(B_0,T)$ only the least-square fit of the data in the T dimension has to be performed resulting in a final 2D-data set $P(B_0,R)$ where the different species are separated by their relaxation rate.

All calculations are performed using the Matlab software package (The MathWorks). The built-in function *lsqcurvefit* of the optimization toolbox was used to perform the least-squares fit. The default stop criterion of the fit routine *(Opt.TolFun)* was set to 10^{-15}. In the case of a magnitude spectrum after Fourier transformation, only positive values for $A_i(v)$ are allowed. The limits for the predefined rate constant are naturally given by the step width and the maximum acquisition time in T. Typically 100 logarithmically spaced rate constants R_i are used to analyze the data set.

A crucial point is the initial guess of the amplitude vector A at every frequency v. Since at each frequency position v_i the contributing relaxation rate is not totally independent from the previous slice at a frequency v_{i-1} the amplitude vector of the previous slice can serve as a starting point for the minimization of the following slice. This speeds up the calculation and results in contiguous line shapes. The least-square fit which converts data from the time domain T into the relaxation rate domain R requires typically about 10 min of computation time on a PC. After inversion the amplitudes for each separated species are projected along the mean value of the respective filter rate.

3. Results and discussions

The echo-detected field-swept spectra of BDPA, TEMPO and CuHis as well as of the mixture are shown in Fig. 1. As can be seen, all three signals overlap in a spectral range of about 100 G. At the marked field position of 3463 G, all three compounds overlap with similar intensities and therefore this magnetic field value was chosen to perform the two-dimensional REFINE experiment.

In a first step the field-swept spectra were separated by the method described above. To encode the relaxation behavior in the new dimension, a saturation recovery sequence was applied followed by a variable recovery time T prior to detection of the EPR signal. In principle every sequence used to measure longitudinal relaxation time could be used, but we specifically chose a saturation recovery sequence to minimize the impact of spectral diffusion. Since the pulsed TWT microwave amplifier limits the maximum length of the saturation pulse to a few microseconds, a picket-fence (PF) sequence was used. Especially for the CuHis complex the PF-sequence minimizes spectral diffusion, which would otherwise lead to non-exponential decay already for a single species [18]. For an optimal perfor-

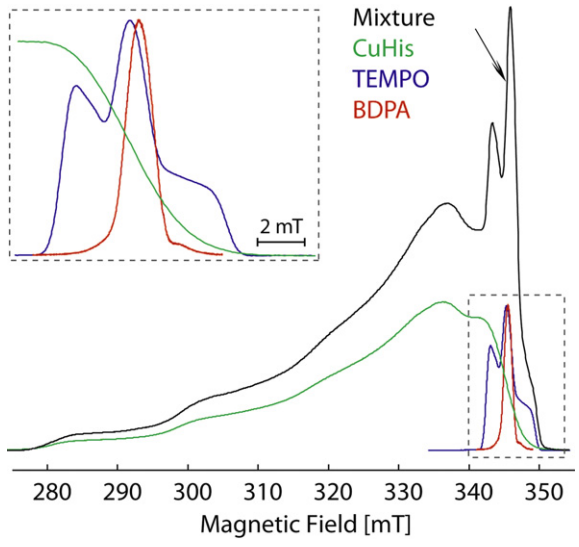

Fig. 1. Field swept EPR spectra of the individual model compounds, CuHis (green), TEMPO (blue), BDPA (red) (colored lines) and of their mixture (black). The dashed line in the inset indicates the field position ($B_0 = 3463$ G) where the 2D-REFINE experiment is performed. For these spectra, 10 scans are taken with 10 averages per point at a shoot repetition time of 15 ms.

mance the pulse separation in the PF-sequence has to be large compared to T_2 to not carry on any coherences. As the pulse separation also has to be small compared to T_1 this technique only works if $T_2 \ll T_1$. The PF-sequence should use maximum power and each pulse should have a nominal flip angle of π. Ideally the pulse train should be as long as T_1, however the maximum gate length, the duty cycle of the TWT as well as the maximum number of pulses allowed by the pulse programmer (31 for the system used) puts some limits on that. The complete sequence to record the two-dimensional data set is given in the inset of Fig. 2.

To demonstrate the capability of the method, only the spectral range (500 G) in which all three compounds overlap was recorded in the field-swept spectrum. As expected for a mixture of paramagnetic species having different spectral shapes and relaxation rates, the observed saturation recovery traces are multi-exponential and vary strongly as a function of the magnetic field.

The analysis of the two-dimensional data set $S(B_0, T)$ was performed using the algorithm as described in the *Data Processing* section, which yields the contributions A_i for every field position. From this the separated field-swept spectra of each individual compound can be constructed. The separated and processed data are shown as a contour plot in Fig. 3 A. After inversion of the experimental data set, three signals with relaxation rates of 0.37, 1.2 and 2.08 kHz can be distinguished. Even for this rather similar relaxation rates, it was clearly possible to separate all three compounds from each other in the relaxation rate dimension. The individual components are identified by comparison with the spectra of the pure compounds.

The fastest relaxing species is the Cu center of the CuHis complex. A slight field dependence of the relaxation rate of this species was observed after exponential fitting (data not shown), indicating some orientation dependence of the obtained relaxation times for this species. The compound with the second fastest relaxing rate in the mixture is BDPA with a rate of 1 kHz under our experimental conditions. The slowest relaxing species is TEMPO, with a longitudinal relaxation rate of only 0.3 kHz. The spectra of the BDPA and the TEMPO samples did not show any relaxation anisotropy over the whole spectral range. In Fig. 3B the individual traces as obtained from the two-dimensional data set (solid lines) are compared on a quantitatively basis with the echo-detected field-swept spectra of the pure compounds (dashed lines). In the high-field region, the separated CuHis spectrum is not reproducing very accurate the pure compound spectra but for TEMPO and BDPA only small differences in the line shape are detected between the spectra obtained from the mixture and the EPR spectra taken of the pure model compounds. This shows that the algorithm used to invert the experimental data set is capable to separate the contribution of the three paramagnetic species. It is possible to unambiguously assign the individual components, despite the fact that the relaxation rates of the three compounds differ only by factors of 2 to 3, but the quality of the inversion data decreases when these factors are small.

Based on these results we performed a two-dimensional REFINE experiment to separate the overlapping hyperfine spectra of the three paramagnetic compounds in the mixture. The experiment was conducted at a fixed field position of 3463 G. At this field position all three compounds overlap (see inset of Fig. 1) and contribute with

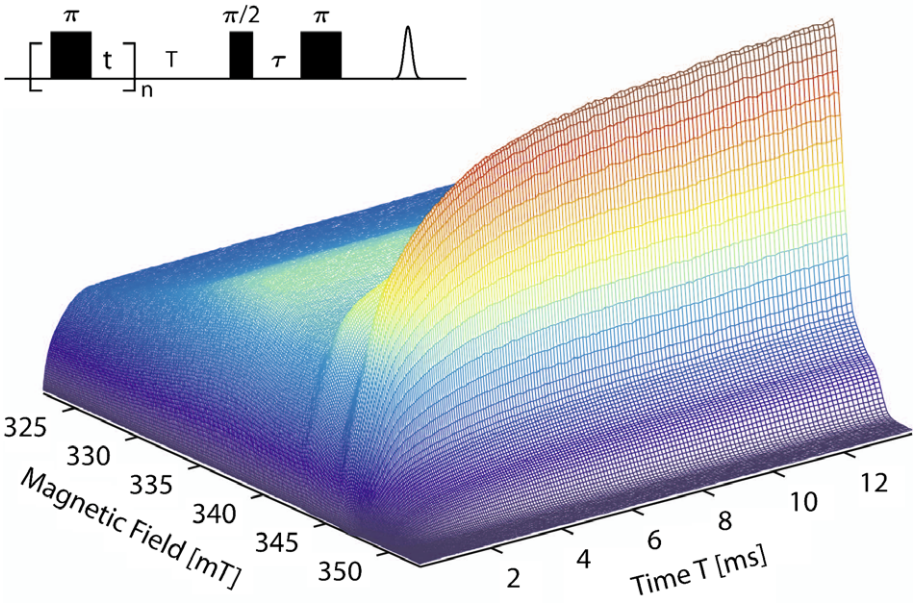

Fig. 2. Saturation-recovery detected field-swept spectrum of the model compounds mixture. The pulse sequence used is shown in the upper left. The saturation is achieved using a picket-fence sequence. The field sweep range was $\Delta B = 500$ G (300 pts) and in the second dimension, the time T was varied between 0 and 14 ms (200 pts). A single scan is taken with 10 shoots per data point at a repetition rate of 15 ms.

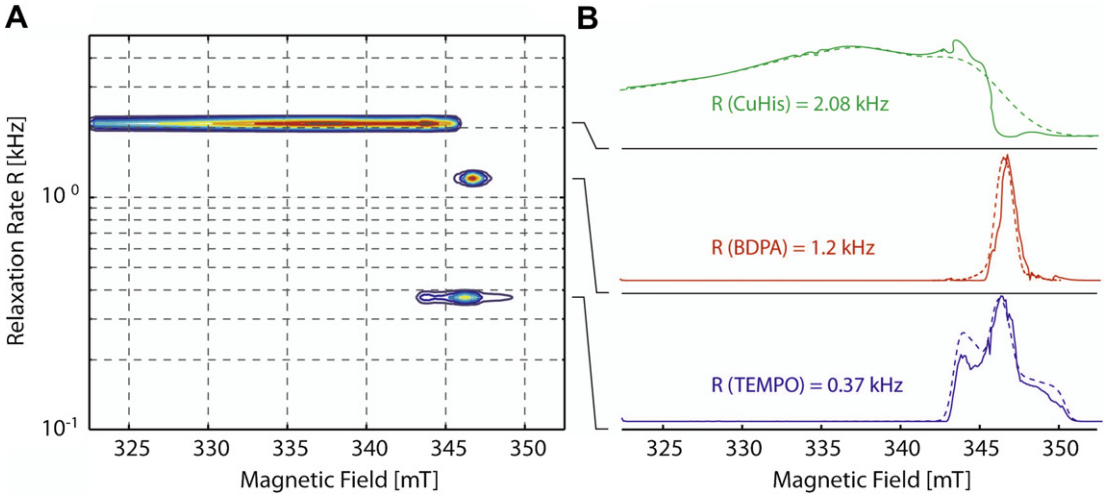

Fig. 3. Inversion of the saturation-recovery detected field-swept spectrum. (A) Contour plot of the spectrum obtained after inversion and projection of the amplitudes to the mean value of the relaxations rates. (B) Individual slices taken from the contour plot at the indicated relaxation rates (solid lines). The obtained field-swept spectra are compared with the field-swept spectra taken of the pure model compounds (dashed lines).

similar amplitudes to the overall EPR signal. A two-dimensional data set was recorded by varying both separation times T and in the pulse sequence (PF–T–$\pi/2$–τ–π). The obtained data set is shown in Fig. 4. After Fourier transformation of the data set along the τ dimension, the relaxation encoded two-pulse ESEEM spectrum is obtained in this dimension. Exponential least-square fitting along the T dimension yields the separated hyperfine (ESEEM) spectra. Three different hyperfine spectra can be distinguished after this procedure. The obtained relaxation rates are 0.25, 0.75 and 2 kHz, similar to the field-swept experiment (Fig. 5). Therefore the three different hyperfine spectra can be easily assigned by their relaxation rates.

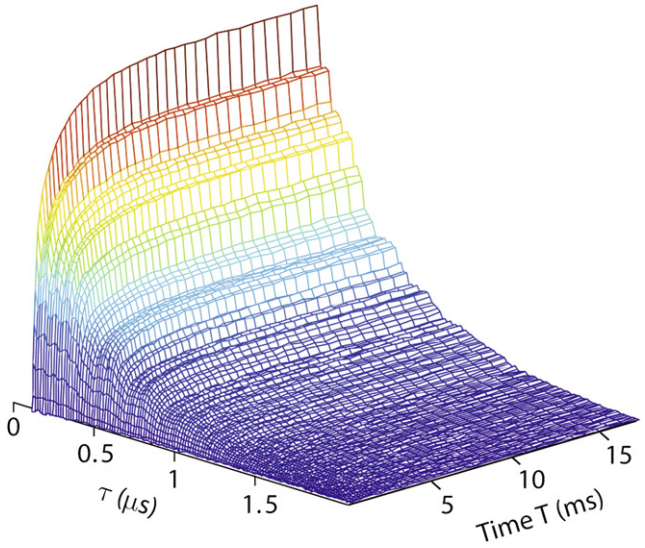

Fig. 4. Two-dimensional REFINE experiment. The spectra is recorded at a magnetic field position of 3463 G. Experimental parameters: $\tau = 0.12$–2.2 μs (500 pts), $T = 0$–17.5 ms (256 pts). A single scan is taken with 10 shoots per data point at a shot repetition time of 18 ms.

In the hyperfine dimension both BDPA and TEMPO samples show single quantum (sq, ~14 MHz) and double quantum peaks (dq, ~28 MHz) indicating a proton electron hyperfine interaction as expected for these compounds (Fig. 5). For the CuHis complex, which was crystallized from deuterated water, several features at frequency of 2–5 MHz are observed. The two main features, occurring at 2.15 and 4.4 MHz are assigned to a hyperfine interaction between the electron and the remote histidine nitrogen ($^{14}N(\delta)$) and to deuterium nuclei from the crystal water, respectively. Only very small intensities for proton peaks at 14 and 28 MHz were observed for the CuHis complex. Again, the three hyperfine spectra as obtained by the two-dimensional REFINE experiment are compared on a quantitative basis with the hyperfine spectra obtained for the pure compounds (Fig. 5B). The REFINE-ESEEM spectra of all three compounds show a very good agreement with the hyperfine spectra of the pure compounds measured individually. Especially the amplitude ratios of the sq and dq peaks are obtained correctly in the REFINE experiment. From Fig. 5B the contributions of the BDPA and the TEMPO sample can clearly be distinguished by the different intensity ratio of sq to dq proton peaks. The weaker and much broader proton hyperfine coupling of the methyl protons, which show up symmetrically around the free proton Larmor frequency for the TEMPO radical can also be seen in the REFINE spectra, but with a somewhat lower intensity. As in the saturation-recovery detected field-swept experiment, this comparison demonstrates that the separation of the hyperfine spectra of all three compounds is possible. Since the line width in two-pulse ESEEM spectra is related to the phase memory time T_m of the electron spins, the resolution is lower than for a three-pulse ESEEM experiment. A three-pulse stimulated echo sequence can also be used in REFINE spectroscopy [13], which will increase the spectral resolution of the hyperfine spectra.

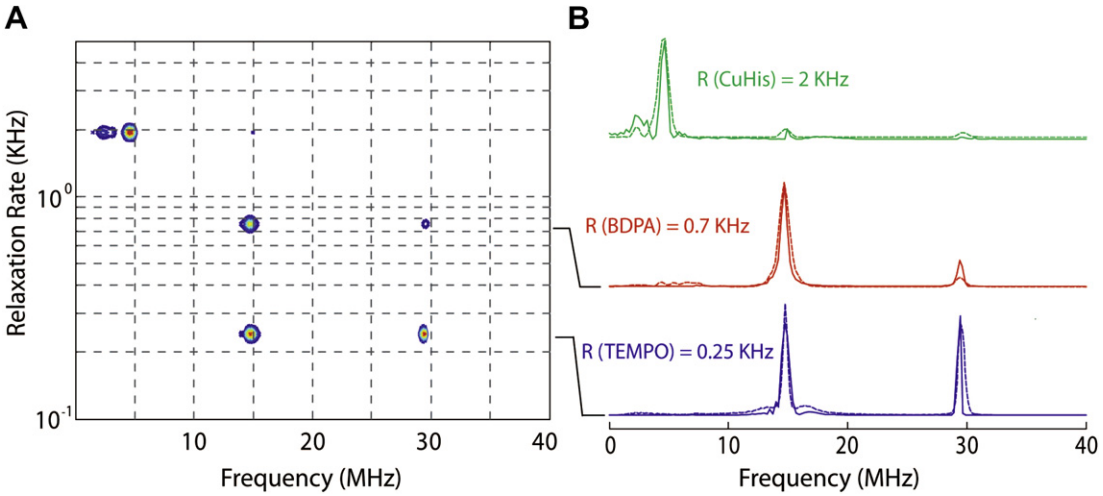

Fig. 5. Inversion of the 2D-REFINE experiment. (A) Contour plot of the spectrum obtained after Fourier transformation along the τ dimension and inverse Laplace transform along the T dimension. (B) Individual slices taken from the contour plot at the indicated relaxation rates (solid lines). The obtained ESEEM spectra are compared with the ESEEM spectra taken of the pure model compounds (dashed lines).

It is difficult to give general rules for the applicability of the REFINE method to real systems. As any multi-dimensional spectroscopy the spectral resolution is only achieved by a prolonged measurement time. Depending on the relaxation rate, reduced signal intensity is achieved in the T dimension, which reduces the signal-to-noise (S/N) ratio as compared to conventional hyperfine spectra. This will mostly affect the slower relaxing species in the mixture. In principle a 3D-REFINE data set $S(B_0, \tau, T)$ would allow to identify the different species and collect in the same experiment the hyperfine spectra of the individual compounds. However, this experiment would usually take too much measurement time. Therefore a field-swept data set $S(B_0, T)$ has to be collected before the two-dimensional REFINE experiment is performed. From this experiment it is possible to assign the different paramagnetic species and to select the optimum magnetic field position(s).

The performance of the presented algorithm was tested using simulated data sets with different numbers of species (up to five) and varying ratios of relaxation rates, amplitudes and noise levels (data not shown). From this the following requirements for a successful separation were deduced and can be used as a guideline for a successful application of a 2D-REFINE experiment:

Signal-to-noise ratio. The S/N ratio has a big influence on the stability of the presented algorithm. For mixtures of up to five different species the S/N ratio in the 2D-REFINE data set has to be >100.

Ratio of relaxation times. The difference of the relaxation times is strongly dependent on the number of components, which have to be separated. In the presence of only two species the difference the ratio can be as small as $T_1^A/T_1^B = 1:2$. In the presence of five different species a ratio of 3–4 is sufficient. In most practical cases, this requirement can be easily fulfilled, especially if different types of components, as for example metal ions from organic radicals, have to be separated.

Number of species. If a S/N of 100 can be achieved and the ratio of relaxation times is 3 or higher the presented algorithm can easily resolve up to five different species.

It should be pointed out, that even with large field dependences of the relaxation rates of some paramagnetic species they can be separated by follow the contours of the individual field-swept spectra.

4. Summary

Using a mixture of three different paramagnetic species, we showed that the field-swept EPR spectra as well as the hyperfine spectra of the individual compounds can be quantitatively separated and assigned if their relaxation times T_1 differ. Using a picket-fence saturation sequence this could be achieved even with relatively small differences in the individual relaxation rates. We have shown that this two-dimensional version of the REFINE experiment allows separating more than two spectrally overlapping species. In this two-dimensional version no pre-knowledge of the relaxation rates of the single compounds is necessary. It can also be applied if the relaxation rates of the single compounds are field dependent and should even work if the relaxation rates of two radicals cross at a given field position. The upper limit of the logarithmic stepped T time window can easily be determined by comparison with a field-swept spectra without inversion pulse. The PF sequence can also be combined with other hyperfine sequences as Davies/Mims ENDOR, three-pulse ESEEM or HYSCORE experiments, as already shown for two compounds. We conclude that this method can be generally applied to systems with several spectrally overlapping paramagnetic species, which so far hindered strongly a quantitative interpretation of hyperfine spectra.

The requirements for a successful separation are reflected by the signal to noise ratio and relative intensity of the individual compounds, the total number of species

and their differences in longitudinal relaxation rates, as stated above. More sophisticated numerical methods are known to solve the Laplace inversion problem and will further improve the quality of the obtained spectra and the robustness of the method. Work on applying this method to complex *I* of the mitochondrial respiration chain with several spectrally overlapping iron–sulfur clusters is under progress.

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