Review

New pulsed EPR methods and their application to characterize mitochondrial complex I

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Abstract

Electron Paramagnetic Resonance (EPR) spectroscopy is the method of choice to study paramagnetic cofactors that often play an important role as active centers in electron transfer processes in biological systems. However, in many cases more than one paramagnetic species is contributing to the observed EPR spectrum, making the analysis of individual contributions difficult and in some cases impossible. With time-domain techniques it is possible to exploit differences in the relaxation behavior of different paramagnetic species to distinguish between them and separate their individual spectral contribution. Here we give an overview of the use of pulsed EPR spectroscopy to study the iron–sulfur clusters of NADH:ubiquinone oxidoreductase (complex I). While FeS cluster N1 can be studied individually at a temperature of 30 K, this is not possible for FeS cluster N2 due to its severe spectral overlap with cluster N1. In this case Relaxation Filtered Hyperfine (REFINE) spectroscopy can be used to separate the overlapping spectra based on differences in their relaxation behavior.

1. Introduction

Paramagnetic molecules, such as organic radicals or metal centers play an important role in biological systems and are in many cases the active centers for electron transfer reactions [1–4]. To study these cofactors, the method of choice is Electron Paramagnetic Resonance (EPR) spectroscopy and the most widely employed technique uses continuous wave (cw) microwave irradiation at a frequency of 9 GHz. In recent years pulsed EPR methods (e.g. ESEEM, HYSCORE, PELDOR) have extended the standard repertoire of EPR techniques and today such hyperfine and dipolar methods can be used to characterize the paramagnetic center itself, its ligand sphere as well as interactions with other EPR active centers up to 8 nm away [5–7].

Unlike NMR spectroscopy, EPR often has single-site resolution since the number of paramagnetic species is limited in the sample. However, a common problem especially in studying biological systems is the presence of more than one paramagnetic species contributing to the overall observed EPR spectrum. This usually results in severe overlap of spectral features from different paramagnetic species, making the analysis of individual contributions difficult and in some cases impossible.

If the paramagnetic species have different g-values, one possibility to separate overlapping spectra is to perform EPR experiments at high magnetic field strengths. Also Electron Nuclear Double Resonance (ENDOR) experiments performed at high magnetic fields (\textgreek{\textless}95 GHz) can dramatically improve and simplify hyperfine spectra [3,8,9]. Unfortunately this advantage does not hold for methods like Electron Spin Echo Envelope Modulation (ESEEM) or Hyperfine Sublevel Correlation (HYSCORE) spectroscopy, since these experiments rely on forbidden transitions, whose transition moments are considerably attenuated at high magnetic fields [10]. In addition, for many metal-based paramagnetic centers, such as iron–sulfur (FeS) centers or hemes, high-field EPR will not be able to separate different signals because of their large g tensor anisotropy.

In most cases, EPR experiments need to be performed at low temperatures due to their fast electron spin relaxation times. However, different paramagnetic species, such as organic radicals and metal-centers or clusters, may exhibit usually large differences in their characteristic relaxation times, especially at low temperatures [11]. Therefore time-domain techniques that exploit differences in the spin-lattice relaxation time ($T_1$) or the phase memory time ($T_2$) will allow distinguishing them. Already the most commonly used two-pulse Hahn-echo or three-pulse stimulated echo sequence provides...
filter capabilities to separate EPR spectra by their $T_2$ or $T_1$ relaxation times [12,13]. This article covers recent developments of pulsed EPR methodology to separate overlapping spectra on the basis of their different relaxation behavior. The focus will be on experiments performed on model compounds to demonstrate the general applicability of such relaxation filters, and first applications to the FeS clusters in complex I of mitochondrial respiration will be shown.

2. Iron–sulfur clusters in complex I

Mitochondrial NADH:ubiquinone oxidoreductase (complex I), the first complex of the respiratory chain, is among the largest and most complicated membrane-bound protein complexes known [14,15]. It links the electron transfer from NADH to ubiquinone with the concomitant translocation of four protons across the inner membrane [16,17]. Because of its central role in respiration, mutations in complex I can lead to a large number of human disorders [18,19]. Furthermore, complex I has been suggested to be a major source of Reactive Oxygen Species (ROS) in mitochondria [20].

The protein complex from mammalian mitochondria is composed of 45 different subunits with a total molecular mass of nearly 1000 kDa [21], but smaller versions can be found in many bacteria [22]. In the obligate aerobic yeast Yarrowia lipolytica, a powerful model system for the structural and functional analysis of complex I [23,24], this enzyme also comprises at least 40 different subunits [25]. Complex I has a typical L-shaped structure, in which the hydrophobic arm is embedded in the membrane and the hydrophilic peripheral part protrudes into the mitochondrial matrix or the bacterial cytoplasm [26–29]. Cw EPR studies have revealed the presence of several paramagnetic cofactors such as iron–sulfur (FeS) clusters and quinones [30–34]. Depending on the organism, complex I hosts up to nine FeS centers [35,36] but not all of them are detectable by EPR because of a diamagnetic oxidation state or electron spin relaxation that is too fast. EPR spectra of Y. lipolytica are similar to those of complex I from bovine heart and Neurospora crassa [24,30]. In its NADH reduced state, the EPR spectra of five FeS clusters are detectable, designated N1 to N5. Cluster N1 is the only EPR detectable binuclear FeS center in complex I, while clusters N2–N5 are tetranuclear FeS clusters [23].

In Fig. 1 the temperature dependence of 9 GHz (X-band) field-sweep spectra of complex I from Y. lipolytica are shown. At 30 K only cluster N1 contributes to the echo-detected EPR spectrum. When lowering the temperature, more and more FeS clusters become visible. At 17 K cluster N1 and N2 contribute equally to the spectrum, while at a temperature of 5 K all five FeS clusters, with their own characteristic g tensor, are detectable [23].

3. Characterization of cluster N1 by hyperfine spectroscopy

At 30 K only cluster N1 contributes to the echo-detected field-sweep spectrum and can therefore be studied individually. The field-swept echo-detected powder spectrum is characterized by the components of the axial symmetric $g$ tensor $g_0$ and $g_\|$, of cluster N1 and the respective orientation selection is shown below. Middle and bottom) Three-pulse ESEEM spectra, taken at field positions corresponding to $g_0$ and $g_\|$. Simulations are shown as dashed lines (parameter given in text). Figure taken from [41].
The hyperfine interactions are partially refocused \[44\]. The observed dq and sq correlation peaks appear as narrow features in the spectrum since the orientation-dependent hyperfine interactions are partially refocused \[44\]. The observed hyperfine couplings are in the typical range for -protons of cysteins ligating FeS clusters \[45,46\].

4. Separation of two spectrally overlapping species

At a temperature of 17 K the 9 GHz EPR spectrum of complex I from \textit{Y. lipolytica} shows contributions from both clusters, N1 and N2 (Fig. 1). In this case, special spectral editing techniques have to be used, to study both clusters individually by EPR spectroscopy. Here, an inversion-recovery filter (IRF) allowed the separation of N1 and N2 by their differences in the \(T_1\) relaxation times.
The pulse sequence for an inversion-recovery experiment is given in Fig. 5. After the initial $\pi$ inversion-pulse the non-Boltzmann polarization of the electron spin will relax back to its thermal equilibrium magnetization with its characteristic longitudinal relaxation time $T_1$. During this process the macroscopic magnetization traverses a zero-crossing point (Fig. 6, top). In a mixture of two species, with different relaxation times $T_1^1$ (slow) and $T_1^2$ (fast), each species will traverse its own zero-crossing point at a filter time of $T_1^1$ or $T_1^2$, as indicated in Fig. 6 by the arrows. Therefore, only the EPR spectrum of the fast or slow relaxing species is detected if the field-sweep spectrum is recorded with the filter time set to either $T_1^1$ or $T_1^2$. This technique shows the EPR spectra of the individual compounds when applied to a mixture of two species. However, in a more complex mixture of several paramagnetic species, this technique can still be useful to simplify crowded spectra by suppressing one species.

The efficiency of such a filter has been tested using a mixture of two model compounds BDPA/PS (slow) and TEMPO/PS (fast) in polystyrene (Fig. 6). The 9 GHz EPR spectrum of the mixture is shown in Fig. 6 (top, black). Using a filter time of $T_{\text{F,BDPA}} = 9 \mu$s or $T_{\text{F,TEMPO}} = 192 \mu$s the individual spectrum of TEMPO/PS or BDPA/PS is observed. For the species with the longer relaxation time (BDPA/PS), the inverted signal is obtained, with a signal intensity of 35% of the maximum intensity. Because of the much shorter relaxation time of TEMPO/PS almost 100% of the maximum signal intensity is observed for the fast relaxing species. A comparison of spectra obtained from the mixture with spectra of the pure compounds shows an excellent agreement.

The inversion-recovery filter can be combined with every pulse EPR sequence for hyperfine spectroscopy such as ESEEM, HYSCORE or ENDOR. This technique is called Relaxation Filtered Hyperfine (REFINE) spectroscopy and some examples of pulse sequences are shown in Fig. 5. In general, the first pulse of the original pulse sequence is applied after the filter time $T_F$. Short filter times can create unwanted echoes that interfere with the measurements (13 for three-pulse REFINE-ESEEM), but they can be removed using an appropriate phase cycle [47].

In complex I, two nitrogen-containing amino acid residues had been suggested as possible candidates for the fourth ligand of iron-sulfur cluster N2 [48,49]. To test this hypothesis, cluster N2 was studied by X-band ESEEM spectroscopy, a difficult task due to the severe spectral overlap with cluster N1. Furthermore, the linewidths in an ESEEM experiment are very sensitive to temperature; therefore, observed differences in the 9 GHz ESEEM spectra taken at 30 and 17 K could easily be misinterpreted. To overcome this problem, IRf field-sweep and REFINE-ESEEM experiments were performed. In Fig. 7 (top panel) IRf field-sweep spectra of complex I taken at 17 K are shown. Using filter times of $T_F = 68$ ns and $420$ ns allowed recording of spectra of cluster N1 and N2 separately. The same filter times can then be used in a REFINE-ESEEM experiment to study the hyperfine interactions of each FeS cluster individually. Such ESEEM time traces and their respective Fourier transformations are shown in Fig. 7 (middle and bottom panel). For comparison, an ESEEM experiment was also performed, using a filter time of $T_F = 50 \mu$s, at which the system is back at the thermal equilibrium polarization. Since the ESEEM spectra at $T_F = 50 \mu$s and $T_F = 68$ ns are similar, it can be concluded that only cluster N1 shows a hyperfine interaction with a $^{14}$N nucleus and that cluster N2 is not ligated by a nitrogen-containing amino acid residue [50]. This was also seen later in the crystal structure of the hydrophilic domain of complex I that revealed a four-cysteine ligation of this cluster [36].

REFINE spectroscopy can also be applied to two-dimensional hyperfine methods, such as HYSCORE. In Fig. 8 the application of REFINE-HYSCORE to a mixture of a copper-histidine complex (CuHis) and BDPA in polystyrene (BDPA/PS) at a microwave frequency of 9 GHz is shown. Without a filter sequence, several correlation peaks...
are observed in the $^{14}$N-region (CuHis) and the $^1$H (BDPA) region of the HYSCORE spectrum (Fig. 8,A). Using the correct filter times to suppress either contributions of CuHis ($T_F^{\text{CuHis}} = 10 \, \mu s$) or BDPA/PS ($T_F^{\text{BDPA/PS}} = 850 \, \mu s$), it is possible to record a HYSCORE spectrum where only the $^1$H correlation peaks of BDPA/PS (Fig. 8,B) or the $^{14}$N correlation peaks of the CuHis complex (Fig. 8,C) are visible. The

![Fig. 7.](Top panel) 9 GHz Inversion-recovery filtered echo-detected field-sweep EPR spectra of complex I of Y. lipolytica at 17 K taken with filter times as indicated (simulations shown as dashed lines). Left: $T_F = 50 \, \mu s$ (no filter effect). (Middle) $T_F = 420 \, \text{ns}$ to select cluster N2 selectively. (Right) $T_F = 68 \, \text{ns}$ to select cluster N1 selectively. (Middle panel) 9 GHz REFINE-ESEEM (three-pulse) time traces recorded with filter times as indicated. Spectra are recorded at a field position as indicated by the arrow (upper left spectrum). (Bottom panel) Fourier transform of the REFINE-ESEEM time traces shown above. Figure taken from [50].

![Fig. 8.](49 GHz $^{14}$N and $^1$H REFINE-HYSCORE spectra recorded from a mixture of a CuHis and BDPA, $T = 20 \, \text{K}$. Upper row: $^{14}$N-region (0–5 MHz), lower row, $^1$H-region (10–20 MHz). All surface plots are shown at the same contour level. (A) HYSCORE without filter sequence. (B) REFINE-HYSCORE with $T_F = 10 \, \mu s$. (C) REFINE-HYSCORE with $T_F = 850 \, \mu s$. All spectra are taken at a field position corresponding to $g = 2$. Figure taken from [61].)
suppression of the $^{14}$N contributions at a filter time of $T^\text{off} = 10$ μs is about 95%, while at a filter time of $T_{1/2}^{BPDPA/PS} = 850$ μs the suppression of $^1$H contributions is almost 100%.

In a recent application of X-band REFINE-HYSCORE to biological systems, the method was used to study the nickel center of the active site of methyl-coenzyme M reductase (MCR) and to characterize the coordination sphere of two reduced paramagnetic states individually [51]. In another example, REFINE was used to study the cobalt bound to myoglobin. Here, two species had to be separated and the coordination spheres of each cobalt were studied by Q-band (35 GHz) REFINE-ENDOR [52]. This example demonstrates that even if the relaxation process shows a large anisotropy across the EPR line, a complete separation is possible (Fig. 19 in [52], supporting information).

5. Separation of several spectrally overlapping species

Using the concept of REFINE, it is possible to separate more than two spectrally overlapping species [53]. For this, the experiment has to be extended into a further dimension, which encodes the relaxation behavior of the individual species either by $T_1$ or $T_2$. If the paramagnetic species show a difference in their relaxation behavior, an inverse Laplace transformation (ILT) along this dimension leads to a separation of the different contributions. Since the numerical ILT is an ill-conditioned problem [54], a robust fitting algorithm, similar to those used in DOSY spectroscopy [55] can be used to perform the task [53,56].

The concept of two-dimensional REFINE is illustrated using the example of a REFINE-ESEEM experiment. The data set obtained in this experiment can be described by the following integral

$$S(\tau, T) = \int I(\nu, R) e^{i2\pi G(\nu, T)d\nu}dR,$$

with $S(\tau, T)$ the two-dimensional experimental data set, $\tau$ the evolution time of the ESEEM experiment, $T$ the separation time in the relaxation domain, $\nu$ the relaxation rates and $I(\nu, R)$ the desired REFINE-ESEEM spectrum with $\nu$ the intensities of the ESEEM spectrum obtained after Fourier transformation and $\nu$ the ESEEM frequencies. $G(\nu, T)$ is called the relaxation kernel, a function that describes the relaxation process. For example for a saturation recovery experiment $G(\nu, T)$ is of the simple form $G(\nu, T) = 1 - e^{-\nu T}$. In the example described above, the desired REFINE-ESEEM spectrum $I(\nu, R)$ is obtained from $S(\tau, T)$ through inverting Eq. (1) by the following procedure:

$$S(\tau, T) \xrightarrow{\text{ILT}} I(\nu, R).$$

Here, prior to the inverse Laplace transformation, an inverse Fourier transformation has to be performed along the spectral dimension of the data set, including standard procedures such as appodization, linear prediction and zero-filling [57]. The result is a two-dimensional spectrum in which the spectral amplitudes are projected along the relaxation rate of the individual species. In the case of EPR or ENDOR spectra, that do not require a Fourier transformation, only the inverse Laplace transformation has to be performed.

The performance of two-dimensional REFINE spectroscopy is demonstrated on a simulated data set of five different species. Without any loss of generality, a field-sweep spectrum is chosen for the spectral domain while the relaxation domain is encoded by an inversion-recovery sequence. The five different species have (arbitrarily chosen) relaxation times of $T_1 = 1, 4, 16, 64, 256$ μs and $g$ values similar to the five iron–sulfur clusters observed in complex I (see caption of Fig. 1) [23]. Furthermore, for a realistic analysis 1% random noise was added to the simulated data set. The result of the analysis is shown in Fig. 9. After inverting the data set, five different species can be clearly identified (Fig. 9, left). The individual EPR spectrum of each species is obtained after projecting the signal amplitudes of each separated species along the mean value of the respective filter rate (Fig. 9, right). By varying factors such as number of species, signal amplitudes or amount of noise added, the following requirements for a successful separation were deduced:

1. **Signal-to-Noise ratio**: For mixtures of up to five different species the S/N ratio in the experimental two-dimensional REFINE data set has to be $\geq 100$.
2. **Ratio of relaxation times**: In the presence of only two species the ratio can be as small as $T_1^1/T_1^2 = 1:2$. If more species have to be separated, a ratio of 3–4 is sufficient. If different kinds of paramagnetic species are present (organic radicals, metal centers), this requirement is usually easily fulfilled.
3. **Number of species**: For $S/N > 100$ and a ratio of relaxation times of $> 3$, up to five species can be resolved easily.

It should be pointed out, that even with a large field dependence of the relaxation rates, caused, for example, by anisotropic librations [58], a separation is still possible, by following the contour lines in the individual field-sweep spectra.

In Fig. 10, an example of two-dimensional REFINE spectroscopy performed at X-band frequencies is shown. Here, the field-sweep spectra as well as the ESEEM spectra of a mixture of three spectrally overlapping components (CuHis, BDPA/PS and TEMPO/PS), which served as model system, are separated based on their different relaxation behavior. Fig. 10 (top, left) shows a two-dimensional saturation-recovery detected field sweep spectrum. 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 these 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 (shown in green). The compound with the second fastest relaxation rate in the mixture is BDPA (shown in red), while the slowest relaxing species is TEMPO (shown in blue) under the given experimental conditions. In Fig. 10 (top, right), the individual traces, as obtained from the two-dimensional data set, are quantitatively compared with the echo-detected field-swept spectra of the pure compounds. In the high-field region, the separated CuHis spectrum is not reproduced very accurately, but the agreement is excellent for TEMPO and BDPA. Based on these results, a two-dimensional REFINE-ESEEM experiment was performed, to separate the overlapping hyperfine spectra of the three paramagnetic compounds. The experiment was conducted at a fixed field position of 346.3 mT. At this field position all three compounds overlap and contribute with similar amplitudes to the overall EPR signal. After inversion of the data, the relaxation encoded two-pulse ESEEM spectra are obtained in this dimension and three different hyperfine spectra can be distinguished (Fig. 10 bottom, left).

For both the BDPA and the TEMPO sample, transitions at around 14 and 28 MHz are observed. These are assigned to sq and dq $^1$H transitions, indicating a proton electron hyperfine interaction as expected for these compounds. For the CuHis complex, which was crystallized from deuterated water, several $^{14}$N and $^2$H resonances were observed. Again, the three hyperfine spectra obtained by two-dimensional REFINE-ESEEM are quantitatively compared to hyperfine spectra recorded from the pure compounds (Fig. 10 bottom, right). All REFINE-ESEEM spectra show very good agreement with the hyperfine spectra of the pure compounds measured individually. In particular
the amplitude ratios of the sq and dq peaks are conserved in the REFINE experiment and contributions of each species can clearly be distinguished by the different intensity ratio of sq to dq proton peaks. Also the weaker and much broader proton hyperfine coupling of methyl protons of TEMPO, which show up symmetrically around the free 1H Larmor frequency can be detected in the REFINE spectra. As in the saturation-recovery detected field-sweep experiment, this comparison demonstrates that the separation of the hyperfine spectra of all three compounds is possible.

Preliminary results of two-dimensional REFINE applied to EPR spectra of complex I are shown in Fig. 11. The experiment was performed at a temperature of 12 K at which cluster N1, N2 and N4 contribute to the EPR signal. The relaxation domain was encoded using a picket-fence saturation sequence [53] and the inversion of experimental data set was achieved using the DISCRETE software package [59,60]. After inversion of the data set, three different species can be identified in the relaxation-encoded dimension. All three show a significant relaxation anisotropy as indicated by the dashed lines in Fig. 11 (right). After projecting the intensities of each species along the mean value of the respective filter rates, the EPR spectrum of cluster N1, N2 and N4 is obtained and compared with simulated EPR spectra of the individual Fe5 cluster and is in exactable agreement given the complexity of the system.

Currently the main limiting factor for the application of two-dimensional REFINE spectroscopy is the available algorithms for inversion of the experimental data set. However, in the future more sophisticated algorithms such as Tikhonov regularization will help to make this task more stable and therefore more widely applicable.

6. Summary and outlook

Pulsed EPR spectroscopy can help to understand the structure and function of complex biological systems that host paramagnetic centers, such as complex I of the mitochondrial respiratory chain. While a crystal structure gives a rather static picture of the architecture, EPR spectroscopy can give more detailed information about paramagnetic centers in their functional states. EPR reveals the identity of paramagnetic co-factors and gives detailed information about paramagnetic centers in their functional states. EPR reveals the identity of paramagnetic co-factors and gives detailed information of about paramagnetic centers in their functional states. EPR reveals the identity of paramagnetic co-factors and gives detailed information of paramagnetic centers in their functional states. EPR reveals the identity of paramagnetic co-factors and gives detailed information of paramagnetic centers in their functional states. EPR reveals the identity of paramagnetic co-factors and gives detailed information of paramagnetic centers in their functional states.

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