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ESEEM studies of the iron-sulphur clusters of succinate dehydrogenase in *Arum maculatum* spadix mitochondrial membranes

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Abstract

We have performed ESEEM spectroscopy in order to obtain structural information about the environment of the [2Fe-2S] cluster and the [3Fe-4S] cluster of succinate dehydrogenase (Centres 1 and 3, respectively) in intact *Arum maculatum* mitochondrial membranes. Both iron-sulphur clusters showed modulations indicative of ^{14}N in the three-pulse echo decay envelopes. We have estimated the hyperfine couplings for the reduced [2Fe-2S] cluster ($A \sim 1.1$ MHz) and the oxidised [3Fe-4S] cluster ($A \sim 0.8$ MHz). Our results are compared with ESEEM data obtained for purified [2Fe-2S] cluster-containing proteins, namely *Spirulina platensis* ferredoxin, a protein for which the three-dimensional structure is known, and *Escherichia coli* fumarate reductase. The hyperfine and quadrupolar coupling parameters determined are consistent with a weak interaction of Centre 1 and Centre 3 with peptide ^{14}N, rather than ^{14}N of imidazole rings.

Key words: Mitochondrion; Complex II; Succinate dehydrogenase; Ferredoxin Fumarate reductase; EPR, pulsed; ESEEM; (Plant); (*S. platensis*)

1. Introduction

We have carried out a feasibility study of the application of electron spin-echo envelope modulation (ESEEM) spectroscopy, to the iron-sulphur proteins in mitochondrial membranes [1]. ESEEM is a pulsed EPR method which is particularly sensitive for observing weak interactions of electron spins with quadrupolar nuclei such as ^{14}N [2], which are difficult to observe by continuous-wave ENDOR. It has been applied to a number of ferredoxins [3,4] and other purified iron-sulphur proteins [5–10]. In this paper we describe an investigation of Complex II, in intact mitochondrial membanes.

Complex II (succinate:ubiquinone oxidoreductase) is a membrane-bound enzyme that couples the oxidation of succinate to fumarate, to the reduction of quinone to quinol [11]. The major component of Complex II is succinate dehydrogenase (SDH, EC 1.3.99.1), which occurs as a membrane-bound enzyme of the tricarboxylic acid pathway. In addition to SDH, *Escherichia coli* has been shown to produce the enzyme fumarate reductase (FRD), which catalyses the reverse reaction [12]. Although these two proteins show extensive amino acid sequence homology, with many similarities in structure, function and organisation, they are immunologically distinct [13].

SDH consists of two hydrophilic subunits: a flavoprotein subunit (Fp) that contains a covalently attached flavin, designated SdhA, and a smaller iron-sulphur protein (Ip) subunit, designated SdhB [14]. These two subunits together form the catalytic domain of the protein, and can be isolated from the membrane together with two smaller hydrophobic peptides (SdhC and SdhD) as Complex II [15]. A third type of prosthetic group, cytochrome *b*, is usually associated with either one or both of the hydrophobic subunits, conferring reactivity with quinones [16–18].

Over the last decade three distinct types of iron-sulphur cluster have been identified in the most intact preparations of SDH and FRD. These are termed Centres 1, 2 and 3. Centre 1, a [2Fe-2S] cluster, is

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Abbreviations: c.w., continuous-wave; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; FRD, fumarate reductase; FT, Fourier Transform; MCD, magnetic circular dichroism; SDH, succinate dehydrogenase; SMP, submitochondrial membranes.

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diamagnetic and EPR-silent in the oxidised state. Upon reduction with either succinate or dithionite, the cluster exhibits an EPR-active $S = 1/2$ ground spin-state, exhibiting a rhombic EPR signal in animal mitochondria at $g_{zyx} = 2.03, 1.935, 1.905$. The EPR ($g_{av} = 1.96$, in common with ferredoxin-type [2Fe-2S] clusters) and redox ($E_m = -7$ mV [19]) properties of Centre 1 make it quite distinct from any other biological [2Fe-2S]$^{2+,1+}$ cluster [11]. Although the higher redox potentials observed with the Rieske clusters were thought to be due to noncysteinyl coordination of the [2Fe-2S] cluster, spectroscopic and site-directed mutagenesis studies suggest that Centre 1 is coordinated by four cysteines [5–7,20–22]. Although ESEEM patterns characteristic of a nitrogen nucleus have been detected, analysis of the hyperfine coupling parameters suggest that the ESEEM most likely arises from a distant peptide nitrogen with a weak superhyperfine coupling ($A \sim 1.1$ MHz) to the unpaired electron spin of Centre 1 [6,21].

Spirulina platensis [2Fe-2S] ferredoxin gives a very similar ESEEM pattern [20,21]. The X-ray structure of the ferredoxin indicates the presence of hydrogen bonds between peptide nitrogen atoms, which may be those detected by ESEEM, and the bridging sulphur atoms [23].

Up until 1985 the existence of a cluster termed 'Centre 2' was controversial. The combination of low temperature MCD spectroscopy and EPR provided the first definitive evidence for the existence of Centre 2, which becomes paramagnetic only on dithionite reduction. The features observed by MCD and EPR spectroscopy have identified Centre 2 as a reduced [4Fe-4S] cluster [24–26]. The characteristic isotropic EPR signal at $g = 2.01$ from oxidised Centre 3 is observed below 21 K. The first indication that Centre 3 was a [3Fe-4S]$^{1+}$ cluster came from a LEFE study, when Ackrell et al. [5] applied the technique to bovine heart Complex II. This result was supported by MCD studies [11,25]. Furthermore, an ESEEM study of Centre 3 in isolated bovine heart Complex II has shown low-frequency features indicative of nitrogen [5].

Our preliminary studies with bovine heart mitochondrial membranes demonstrated that it was possible to observe ESEEM from oxidised Centre 3, the only centre in the membranes which is paramagnetic in the oxidised state. However, it proved difficult to selectively reduce SDH Centres 1 and 2, the spectra of which overlap completely with the signals from the iron-sulphur centres of mitochondrial Complex I and Complex III in the $g = 2$ region of the EPR spectrum. Therefore, we made use of mitochondrial membranes from the mature spadix of the plant *Arum maculatum*. EPR studies of the respiratory chain of these mitochondria revealed that they were rich in iron-sulphur clusters [19,27]. The redox potentials of these clusters were similar to those of Complex II of mammalian mitochondria, and the EPR signals of Complexes I and III were almost absent from the mitochondria [27].

Mitochondria from *A. maculatum* spadices are thus a useful material for examination of the iron-sulphur clusters of higher eukaryotic SDH, virtually free from the overlapping signals of other iron-sulphur proteins. Here we have used them for the study of the electron spin-echo spectroscopic properties of Complex II in the mitochondrial membrane. The results of these measurements have been compared with those obtained previously from purified bovine heart Complex II [6] and purified *E. coli* FRD [6,7,20].

2. Materials and methods

A. maculatum inflorescences were collected from the wild whilst in the thermogenic γ-stage. Mitochondria were prepared from the spadices as described by Cammack and Palmer [19]. Submitochondrial membrane particles (SMP) were prepared, and concentrated by centrifugation into quartz EPR tubes, as described previously [1]. *Spirulina platensis* ferredoxin was purified by the method described by Hall et al. [28].

2.1. EPR sample preparation, for the specific observation of the iron-sulphur clusters of Complex II in SMP

The enzymic activity of SDH in isolated mitochondria is suppressed by tight binding of the competitive inhibitor oxaloacetate [29]. Any oxaloacetate already bound to the protein remains bound through the procedure used to prepare submitochondrial particles. Before reducing with succinate, it was therefore necessary to first activate the SDH centre of Complex II. This was achieved by incubating the membranes with 5 mM $MgCl_2$, 1 μM/mg protein ATP and 1 μM/mg protein KH_2PO_4 for 10 min. at room temperature, under argon. The membranes, concentrated in EPR tubes, were then selectively reduced for the iron-sulphur clusters of SDH, either partially with 30 mM succinate for 15 min, or fully with 2 mM dithionite for 2 min. under an argon atmosphere.

2.2. Pulsed EPR measurements

All pulsed measurements were recorded on a Bruker ESP380 spectrometer (ESP380 software) fitted with an X-band dielectric variable Q resonator (DlQ-H8907) loaded with a sapphire annulus, and an Oxford Instruments liquid helium cryostat (CF 935) and ITC-4 temperature controller. The ESEEM data were collected at approx. 4 K using a three-pulse (90°-τ-90°-T-90°) sequence, as described previously [7,20,30].

3. Results

3.1. c.w. EPR spectral features of A. maculatum mitochondrial membranes

Fig. 1 shows the c.w. EPR spectra of the *A. maculatum* spadix SMP, subsequently used for the ESEEM measurements. The membranes were redox poised for the specific detection of SDH, using either fully oxidised membranes for Centre 3 or membranes reduced selectively with the Complex II substrate, succinate, for Centre 1. The EPR signals most clearly observed arose from iron-sulphur Centres 1, 2 and 3 of Complex II SDH. The nature of the paramagnetic species giving rise to the EPR signal(s) observed are commented on below.

The EPR spectrum of oxidised membranes (as prepared) showed a relatively isotropic, rapidly-relaxing signal centred at $g \sim 2.01$ in the 6–18 K temperature range (Fig. 1a). This signal is typical of a [3Fe-4S] cluster and has been assigned to Complex II Centre 3 (Table 1). On increasing the temperature to ≥ 20 K the [3Fe-4S] cluster signal was no longer observed; instead, a new axial signal at $g_{z,xy} = 2.16, 2.015$ typical of oxidised cytochrome aa_3 oxidase copper was observed. In addition, another weaker signal was observed to low-field with features at $g \sim 2.40$ and 2.25, from low-spin ferric cytochrome *P*-450 [31]; this signal was only clearly detected upon vertically expanding the scale of the EPR spectrum.

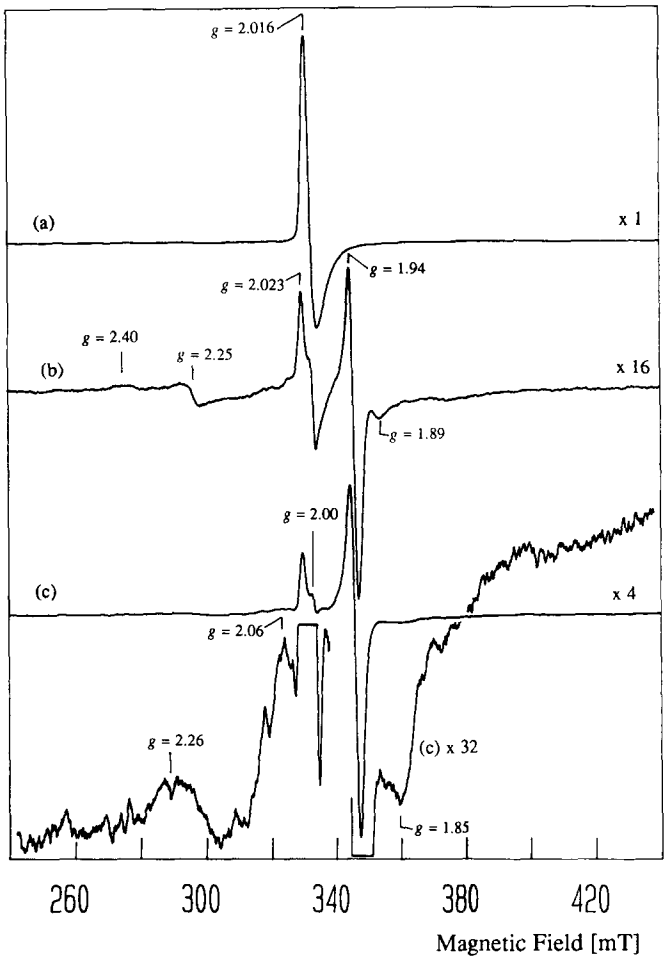

Fig. 1. c.w. EPR spectra of *Arum maculatum* SMP. (a) oxidised, (b) reduced with succinate, and (c) reduced with dithionite. Conditions of measurement: microwave power, 20 mW; microwave frequency, 9.33 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; time constant, 0.328 s; sweep rate, 2.384 mT/s; temperature, (a, c) 6 K, (b) 18 K; number of scans, 2.

Table 1
c.w. EPR data for samples of *Arum maculatum* SMP, at 15 K

Treatment	EPR-detectable species	g-factors
None	Centre 3 [a,*]	a. $g = 2.01$
SDH activation, +30 mM succinate, for 15 min	Centre 1 [b] Centre 3 [a, partially reduced]	b. $g_{z,xy} = 2.02, 1.93$
SDH activation, +2 mM dithionite, for 2 min	Centre 1 [b]; Centre 2 [c,*] and Complex I, III	c. $g = 2.25, 2.06,$ 1.98, 1.84

* Detected below 20 K only.

Membranes, partially reduced with succinate (after activation of mitochondrial SDH), exhibited a signal characteristic of the [2Fe-2S] cluster of Complex II Centre 1 at $g \sim 2.02$ and 1.93, together with a free radical signal at $g = 2.00$ (Fig. 1b, Table 1). The latter signal is probably due to SQ_s, the half-reduced form of ubiquinone which is associated with SDH. The signal from low-spin cytochrome *P*-450 persisted after succinate treatment. The strong signals observed from membranes, fully reduced with dithionite, arise predominantly from Complex II Centres 1 and 2 (Fig. 1c). The weak EPR features characteristic of the [4Fe-4S] cluster of Centre 2 were only observed at temperatures of ≤ 20 K (Table 1). The EPR signal from low-spin cytochrome *P*-450 is no longer observed, due to formation of the ferrous form, which is high-spin.

It should be noted that although the dithionite-reduced membranes give rise to a much stronger signal from Centre 1 at $g_z \sim 2.02$ and $g_{xy} \sim 1.93$, this signal is probably superimposed by the [2Fe-2S] cluster signal from the [2Fe-2S] cluster of Complex I, although the latter is present in small amounts. However, reduction of the membranes with succinate has probably resulted in the 'specific' observation of SDH Centre 1.

3.2. ESEEM spectroscopy of A. maculatum mitochondrial membranes

The ESEEM data presented below were obtained by the three-pulse or 'stimulated echo' technique. In this section we describe the application of ESEEM spectroscopy to both oxidised and reduced *A. maculatum* SMP, which predominantly contain SDH, and

minor amounts of the iron-sulphur clusters of Complexes I and III. We have also recorded three-pulse ESEEM spectra of *S. platensis* ferredoxin, a well characterised iron-sulphur protein containing a [2Fe-2S] cluster. Data were obtained at various values of B_0 corresponding to the principal g-factors, as well as other regions within the spin-echo absorption envelopes.

Fig. 2 shows the ESEEM spectrum observed with the [3Fe-4S] cluster of SDH Centre 3 in oxidised *A. maculatum* mitochondrial membranes. The Fourier transform (FT) spectrum obtained at the g-factor of 2.017 shows two lines centred at 3.20 and 4.14 MHz. Other weaker features are detectable over noise level to lower frequency, at ~1 and ~2 MHz. Control measurements, conducted outside the area of Centre 3 absorbance at $g = 2.10$ and 1.65, showed none of the features observed in the area of SDH Centre 3 absorbance.

Fig. 3a displays the three-pulse ESEEM FT spectrum obtained with dithionite-reduced mitochondrial membranes. At low temperature (≤ 20 K) these membranes showed a c.w. EPR spectrum typical of reduced Centres 1 and 2. ESEEM data were obtained using a τ-value of 136 ns at magnetic fields corresponding to $g = 1.93$ and 2.02. At $g = 1.93$, the g_{xy} factor of SDH Centre 1, the FT shows four intense lines in the low-frequency region, at ~0.4, 1.92, 3.15 and 4.45 MHz (Fig. 3a). At the high-frequency end of the spectrum a line at 15.5 MHz was observed, and was assigned to matrix protons interacting with the paramagnetic iron-sulphur cluster(s) (i.e., the distant proton nuclei from the protein or water environment within a sphere with a radius of 6 Å). Decreasing the magnetic field to record the ESEEM spectrum at $g = 2.02$ resulted in slight shifts of the modulating frequencies to ~0.7, ~1.9, 3.20 and 4.40 MHz.

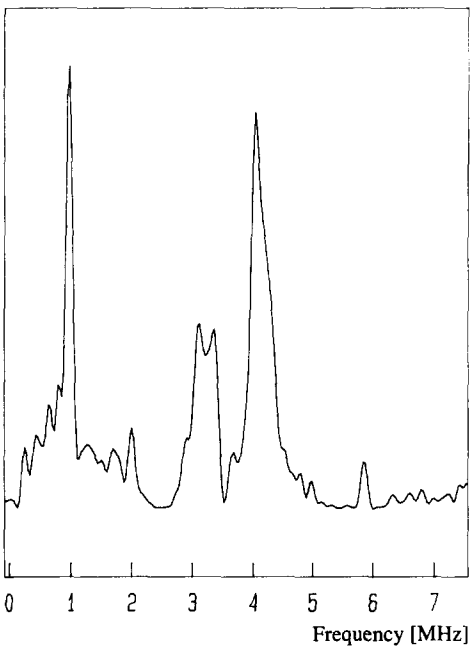

Fig. 2. Three-pulse ESEEM Fourier transform of the oxidised [3Fe-4S] cluster of *A. maculatum* succinate dehydrogenase in intact membranes, at $g = 2.017$. Measurement conditions were: pulse width (for a 90° pulse), 16 ns; bandwidth, 100 MHz; sample temperature, 4 K; τ-value, 136 ns; microwave frequency, 9.70 GHz; high power pulse attenuation, 0 dB; shot repetition time, 10.24 ms; number of shots, 30; video amplifier gain, 36 dB.

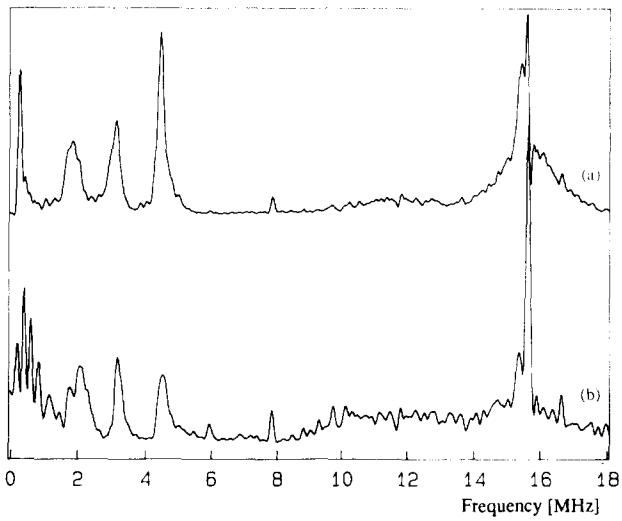

Fig. 3. Three-pulse ESEEM Fourier transform spectra of reduced *A. maculatum* succinate dehydrogenase in intact membranes, at $g_{xy} = 1.93$. The membranes were reduced with either (a) dithionite, or (b) succinate. Measurement conditions were as for Fig. 2, except: sample temperature, 3.8 K; microwave frequency, (a) 9.7254, (b) 9.6978 GHz; shot repetition time, (a) 5.12, (b) 20.48 ms; number of shots, 50; video amplifier gain, (a) 45, (b) 54 dB.

Similar ESEEM spectra were then recorded with membranes partially reduced with succinate (Fig. 3b). The [4Fe-4S] cluster of Centre 2 is EPR-silent in these membranes, whilst the [2Fe-2S] cluster of Centre 1 is still paramagnetic and hence EPR-detectable. At $g_{xy} = 1.93$ the FT spectrum showed lines at ~0.6, 2.10, 3.25, 4.50 MHz. Modulations were clearly detected at $g_z = 2.02$ also, with lines in the FT at 0.6, 2.15, 3.18 and 4.54 MHz. As the spectra are comparable for both partially and fully reduced SDH, it is suggested that Centre 1, the common paramagnetic species in both membrane samples, gives rise to the observed ESEEM.

One of the simplest systems for studying the [2Fe-2S] cluster is the typical plant-type ferredoxin from the algae *S. platensis*. The ferredoxin contains no other iron-sulphur cluster that may contribute to the ESEEM observed, and the three dimensional structure of the ferredoxin has been determined by X-ray analysis to 2.5 Å resolution [23]. The crystallographic data has demonstrated that the [2Fe-2S] cluster of the ferredoxin has four cysteinyl ligands, and that there are several nitrogens from the polypeptide backbone that are close enough to form NH · · · · · S hydrogen bonds

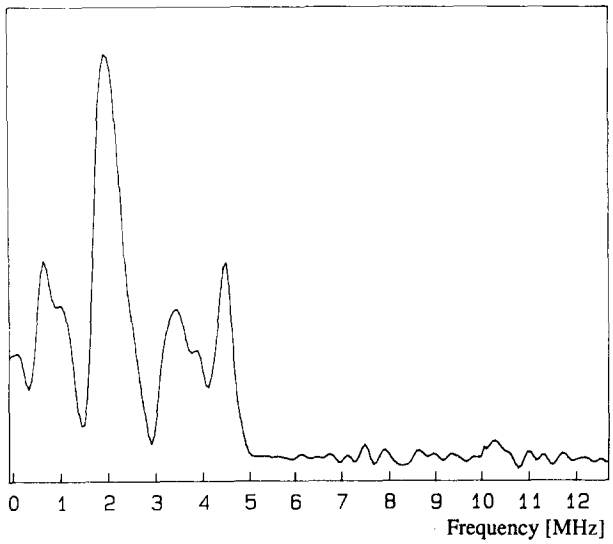

Fig. 4. Three-pulse ESEEM Fourier transform of dithionite-reduced *Spirulina platensis* [2Fe-2S] ferredoxin, at $g = 1.96$. Measurement conditions were as for Fig. 2, except: sample temperature, 3.7 K; microwave frequency, 9.7680 GHz; shot repetition time, 15.36 ms; number of shots, 10; video amplifier gain, 27 dB.

to the cluster. In the reduced state the ferredoxin displayed a rhombic EPR signal, with $g_{xyz} = 1.883$, 1.958 and 2.047. The three-pulse ESEEM FT spectrum obtained at $g_y = 1.96$ with dithionite-reduced *S. platensis* ferredoxin is displayed in Fig. 4, which clearly shows four intense lines centred at 0.79, 1.97, 3.55 and 4.47 MHz.

The three-pulse ESEEM spectra obtained with *A. maculatum* SDH Centres 1 and 3, and *S. platensis* [2Fe-2S] ferredoxin are consistent with coupling to ^{14}N. Previously published ESEEM results of *E. coli* FRD Centre 1 [6,7] have also shown four lines that were assigned to an interaction between the unpaired spin of the [2Fe-2S] cluster and a weakly-coupled ^{14}N nucleus. Similar results have been obtained with Centre 1 of purified bovine heart SDH [21]. Furthermore, we note that the ESEEM spectrum of *A. maculatum* SDH Centre 3 is essentially identical to the spectrum of *E. coli* FRD Centre 3 [30], suggesting a similar environment for the oxidised [3Fe-4S] cluster of FRD and SDH.

The high-frequency lines (between 5 and 8 MHz) attributed to nitrogens coordinated to the Fe^{2+} site of the Rieske [2Fe-2S] cluster [1,10] were not observed with either SDH or the ferredoxin. This evidence argues against nitrogenous coordination of an Fe-atom of the [2Fe-2S] and [3Fe-4S] clusters of SDH. A site-directed mutation of a conserved cysteine to an aspartic acid residue (*Frd*BC65D) has been shown to have no significant effect on the growth rate, activity, redox potential or EPR properties of *E. coli* FRD Centre 1 [32]. Therefore, we cannot exclude partial oxygenic coordination of the [2Fe-2S] cluster of SDH.

The four-line pattern observed with Centre 1 is the result of electron-nuclear hyperfine, nuclear Zeeman and nuclear quadrupole coupling. The positions of these lines on the frequency scale shifted slightly as the ESEEM was examined across the EPR absorbance envelope, due to different orientations of the g matrix relative to B_0. However, the shifts are small because the modulation frequencies are primarily a function of ^{14}N nuclear quadrupole interactions [33]. Previously published ESEEM results of *E. coli* FRD Centre 1 [6,7] have also shown four lines that were assigned to an interaction between the unpaired spin of the [2Fe-

Table 2
Hyperfine and quadrupolar parameters of SDH Centres 1 and 3 in *A. maculatum* SMP, compared with purified *E. coli* fumarate reductase and *S. platensis* ferredoxin

Protein	Cluster	n	Hyperfine and quadrupolar constants (MHz)		Ref.
			A	e^2qQ [a]	
A. maculatum SDH, in SMP					
partially-reduced	[2Fe-2S]	2	1.17 ± 0.11	3.39 ± 0.01	this work
fully-reduced	[2Fe-2S]	2	1.10 ± 0.03	3.32 ± 0.06	this work
oxidised	[3Fe-4S]	2	0.82	3.24	this work
E. coli FRD, purified					
partially-reduced	[2Fe-2S]	5	1.05 ± 0.08	3.44 ± 0.04	this work
fully-reduced	[2Fe-2S]	6	1.06 ± 0.08	3.41 ± 0.10	this work
oxidised	[3Fe-4S]	6	0.6 ± 0.04	3.36 ± 0.07	[30]
	[2Fe-2S]		1.10 ± 0.10	3.30 ± 0.10	[6]
Bovine heart Complex II, purified	[3Fe-4S]		0.48		[5]
S. platensis [2Fe-2S] ferredoxin, purified	[2Fe-2S]	7	1.01 ± 0.14	3.52 ± 0.12	this work

[a] The quadrupolar coupling constant (e^2qQ) was determined using an asymmetry parameter of 0.5. The data shown are expressed as the mean ± S.D., where n represents the number of data sets.

2S] cluster and a weakly-coupled ^{14}N nucleus. Similar results have been obtained with Centre 1 of purified bovine heart SDH [21].

3.3. Interpretation of the modulation frequencies

We have applied the theoretical and graphical analyses of Dikanov et al. [34], in order to analyse the ESEEM frequencies observed with SDH Centres 1 and 3, as described previously [30]. By assuming that the two highest frequency lines of the four-line ESEEM pattern arise from $\Delta m_i = 2$ transitions, we have determined the isotropic hyperfine (A) and quadrupolar (e^2qQ) couplings for *A. maculatum* SDH Centres 1 and 3, and *S. platensis* ferredoxin. These values are summarised in Table 2. The coupling parameters for *A. maculatum* SDH Centre 1 are nearly identical to those determined by a complete simulation involving relative angles [6], for the same centre in purified *E. coli* FRD. It is of course interesting to compare these results to those of *S. platensis* [2Fe-2S] ferredoxin (see Table 2). Although the values are not identical, there is nevertheless a close match between the ESEEM derived coupling parameters of *S. platensis* ferredoxin and FRD and SDH Centre 1.

The modulations observed with bovine heart Centre 3 by Ackrell et al. [5] were assigned to a weak interaction of the centre with ^{14}N in the local environment, with a hyperfine coupling (based on simulation data) of 0.48 MHz. However, the possibility that the ^{14}N interactions were due to flavin, were not completely excluded. The only other ESEEM study of an oxidised [3Fe-4S] cluster has been conducted by Cammack et al. [35] on *Thiocapsa roseopersicina* hydrogenase. The component giving rise to the weak modulation pattern has been assigned as a ^{14}N nucleus, probably from the polypeptide chain. The couplings we have estimated for the [3Fe-4S] cluster of hydrogenase are $A = 0.9$ MHz and $e^2qQ = 3.1$ MHz; these values are similar to those of *A. maculatum* SDH Centre 3.

The e^2qQ values for *A. maculatum* SDH Centres 1 and 3 are almost identical to those obtained with peptide nitrogens ($e^2qQ = 3.0$–3.4 MHz [36,37]). The only other amino acid with an ^{14}N site with equivalently large quadrupolar couplings is the imino nitrogen of histidine ($e^2qQ = 3.36$ MHz with $\eta = 0.13$), though the value of this coupling constant decreases upon coordination to metals [38]. Therefore, the most likely assignment for the low-frequency ESEEM of *A. maculatum* Centres 1 and 3 is coupling between the clusters and a distant ^{14}N of the polypeptide chain.

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