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ESEEM and ENDOR studies of the Rieske iron-sulphur protein in bovine heart mitochondrial membranes

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Abstract

Electron nuclear double resonance (ENDOR) and electron spin echo envelope modulation (ESEEM) were applied to the respiratory-chain iron-sulphur clusters in natural bovine heart mitochondrial membranes. By using specific reduction, signals were observed from the Complex III Rieske [2Fe-2S] cluster. In ENDOR, ^{1}H hyperfine couplings in the range 0.5–7 MHz were observed. In ESEEM, modulations were obtained which were assigned to two ^{14}N nuclei of directly-coupled imidazole ligands. The ESEEM spectra are similar to previous observations on purified iron-sulphur proteins of this type, in which the iron-sulphur cluster is coordinated by two cysteine and two histidine ligands. They confirm that the coordination of the cluster in the purified proteins, with two cysteinyl sulphur and two histidine nitrogens, is unchanged from its natural mitochondrial membrane environment. In order to investigate the possible interaction of the membrane-bound Rieske protein with quinones, measurements were conducted on membranes preincubated with 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT), and in the pH range 6–7.5. No significant changes were detected, either in the proton hyperfine couplings as detected by ENDOR, or in the nitrogen couplings to the histidines as detected by ESEEM.

Key words: Mitochondrion; Complex III; Rieske protein; EPR; ESEEM; ENDOR

1. Introduction

EPR spectroscopy provides a versatile method for observing paramagnetic metal centres in proteins. It has proved especially useful for the discovery and investigation of membrane-bound iron-sulphur proteins, which are difficult to resolve by optical spectroscopy [1–3]. EPR is capable, in principle, of providing detailed information about metal centres, from studies of hyperfine interactions with adjacent nuclear spins.

In the case of frozen solutions of transition-metal centres, the narrow hyperfine structure is obscured by the EPR linewidth. More discriminating techniques are available, such as electron nuclear double resonance (ENDOR) and the pulsed EPR technique, electron spin echo envelope modulation (ESEEM). The latter method is particularly sensitive for observing weak interactions of electron spins with quadrupolar nuclei such as ^{14}N [4], which are difficult to observe by continuous-wave ENDOR. It has been extensively used in studies of metal centres in proteins [5–12], but has not been applied to such proteins in intact membrane systems such as the mitochondrial respiratory chain. This is partly because of the lower sensitivity of the method compared to conventional c.w. EPR, and partly because of the large number of overlapping paramagnetic species (in this case, the iron-sulphur clusters of mitochondrial Complexes I, II and III).

In the conventional first-derivative c.w. EPR presentation it is relatively easy to resolve overlapping spectra by the turning-points in the spectra; by contrast, in pulsed EPR the echo recorded at a particular

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Abbreviations: BSA, bovine serum albumin; c.w., continuous-wave; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; Hepes, *N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulphonic acid]; PDO, phthalate dioxygenase; Q_o-site, quinol oxidation site; SMP, submitochondrial particles; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; UHDBT, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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magnetic field derives from the sum of the absorption spectra of all the species. Moreover, in c.w. EPR it is possible to exploit the relaxation properties of different paramagnetic species. Some centres are observable at higher temperatures than others, and others are readily saturated out by high microwave power at low temperatures. In ESEEM particularly, power saturation does not occur in the same way; and since for iron-containing proteins it is necessary to use the lowest temperatures to obtain a measurably long phase memory time, it is not feasible to use temperature for discrimination.

For these reasons, ESEEM studies have previously been carried out on purified proteins, and, in a few cases, purified respiratory-chain complexes [7,11,12]. However, it is possible that in extracting the proteins from the membrane, the subtle interactions around the metal centres may become distorted. Moreover, there are some interactions, such as with transmembrane potentials and with quinones, which will inevitably be lost when the membranes are disrupted. For these reasons we have carried out a feasibility study for ESEEM of the iron-sulphur clusters of the mitochondrial respiratory chain. In this paper we describe an investigation of Complex III.

The mitochondrial respiratory chain contains a large number of iron-sulphur clusters in Complexes I, II, and III (Centres N1–N4, Centres S1–S3, and the Rieske cluster, respectively), which may be detected by c.w. EPR [1–3,13,14]. The EPR spectra of these clusters tend to overlap, but may be discriminated on the basis of their specific g-factors, temperature dependence and redox potentials [15].

The Rieske [2Fe-2S] cluster protein of Complex III was first described as a component of the cytochrome bc_1 segment [16,17]. It has a characteristic EPR spectrum ($g_{xyz} \sim 1.80$, 1.89 and 2.02), which is distinct from those of other [2Fe-2S] cluster proteins. The term 'Rieske protein' has been applied to iron-sulphur proteins with similar EPR signals, observed in mitochondria [18,19], chloroplasts [20,21] and bacteria [22,23]. It has since been extended to soluble enzymes such as phthalate dioxygenase (PDO) [24].

The Rieske protein has been isolated as a monomeric protein (M_r, 24 000), which contains a single [2Fe-2S] cluster, with a redox potential that is considerably higher ($E_m = +280$ mV, bovine heart) than typical [2Fe-2S] ferredoxins [25]. Biological and spectroscopic studies had, until recently, failed to explain the structural differences between the Rieske [2Fe-2S] cluster and the ferredoxin-type [2Fe-2S] clusters which are coordinated to proteins by four cysteines [26].

The *Thermus thermophilus* Rieske protein [10,26], and *Pseudomonas cepacia* PDO [10,27], have been extensively studied by visible, Mössbauer, EXAFS, EPR, ENDOR and resonance Raman spectroscopies [10–12,26–30]. These studies indicated that each Rieske [2Fe-2S] cluster has at least two non-cysteine ligands. X-band ENDOR studies of these proteins demonstrated that the clusters have nitrogen ligands [10,11]. Further studies using Q-band ENDOR of ^{15}N-histidine-enriched PDO conclusively demonstrated the presence of two magnetically distinct histidine ligands coordinated to the [2Fe-2S] cluster [27]. Analysis of the ^{15}N hyperfine and quadrupolar coupling tensors indicated a roughly tetrahedral coordination at Fe^{2+}, with the N-Fe-N ligand plane corresponding to the g_z-g_x plane.

Further evidence for nitrogen coordination of Rieske centres has been provided by ESEEM studies of isolated complexes of the bc_1 type by Britt et al. [12]. Spectra of the bc_1 complexes of bovine heart mitochondria, *Rhodospirillum rubrum* and *Rhodobacter sphaeroides*, and the b_6f complex of spinach chloroplasts, all indicated a nitrogen coordination environment similar to that observed with the [2Fe-2S] clusters of PDO [27] and the isolated Rieske protein [28].

It is of great interest to extend these observations of the Rieske cluster to intact mitochondrial membranes, since the protein undergoes interactions with quinols, quinones and quinol-type inhibitors [25,31,32]. These interactions affect the protein's EPR spectrum, although the small changes in lineshape and the g-factor shifts are not observed with purified proteins. These interactions of the Rieske cluster with quinols and quinones form the basis for a number of hypotheses about the mechanism of proton translocation in Complex III [25,33]. Such interactions will obviously be dependent on a membrane environment. The Rieske iron-sulphur cluster of Complex III in bovine heart mitochondrial membranes is a suitable subject for study by ESEEM. Its spectrum may be isolated from those of the other iron-sulphur clusters because of its g-factor around 1.80 which occurs at higher field than those of ferredoxin-type [2Fe-2S] clusters. Moreover, its positive redox potential allows it to be reduced under conditions where most of the other iron-sulphur clusters remain non-magnetic.

2. Materials and methods

Preparation of mitochondria

Mitochondria were prepared by a modification of the protocol described by Rickwood et al. [34]. The bovine heart muscle tissue was separated from fat and connective tissue and minced finely. The mince was suspended in an equal volume of high-EDTA buffer (50 mM EDTA, 210 mM sorbitol, 70 mM sucrose, 0.1% BSA, 10 mM Tris-HCl (pH 7.6)), and washed six times. It was then incubated with trypsin (0.5 mg/g tissue) for 30 min, after which trypsin inhibitor (1.5

mg/g tissue) was added and the preparation washed several times. The tissue was homogenised in a small Waring blender. The homogenate was centrifuged at $1500 \times g$ for 5 min, the supernatant filtered through muslin, and recentrifuged at $13000 \times g$ for 10 min. The dark brown pellet was washed in low-EDTA buffer (100 μM EDTA, 10 mM Tris, 225 mM sorbitol, 75 mM sucrose and 0.1% BSA, adjusted to pH 7.6 with HCl), recentrifuged at $13000 \times g$, and the final pellet resuspended in low-EDTA buffer. Typical preparations had a respiratory activity in the oxygen electrode of ~ 80 nmol oxygen reduced/min/mg mitochondrial protein at room temperature with succinate as substrate.

Preparation of mitochondrial membranes

As pulsed EPR experiments require high protein concentrations, the mitochondria were converted to submitochondrial membrane particles (SMP) by sonication. This gave us a preparation of inner and outer mitochondrial membranes (free of matrix enzymes) for the investigation of Complex III. The mitochondria were thawed, diluted to a protein concentration of 10 mg/ml with Medium A (10 mM Na-Hepes, 1 mM $MgCl_2$, 1 mM Na-ATP, 5 mM succinate, 200 μM NADH and 1% BSA (pH 7.1)), and centrifuged at $27000 \times g$ for 10 min. The pellet was resupended to ~ 20 mg protein/ml in Medium A, and 3 ml aliquots sonicated in an ice-bath with eight separate 15-s bursts (with 15-s intervals) at 18 A, using an MSE Soniprep-150 fitted with a microtip probe. The sonicated suspension was centrifuged at $27000 \times g$ for 10 min. and without disturbing the lower mitochondrial pellet, the supernatant was removed and stored at 4°C. The mitochondrial pellet was resuspended, sonicated and re-centrifuged as before. The supernatant obtained was combined with the original supernatant fraction and centrifuged at $130000 \times g$ for 1 h. The viscous brown pellet was washed in sucrose medium (50 mM Na-Hepes, 250 mM sucrose (pH 7.5)) and re-centrifuged as above. For EPR studies the final SMP pellet was resuspended in a minimal volume of sucrose medium.

EPR sample preparation, for the specific observation of the Rieske [2Fe-2S] cluster of Complex III in SMP

To obtain a solid pellet of SMP in the bottom of a quartz EPR tube, perspex blocks especially designed to fit a Sorvall 8×50 ml (SS34) rotor, with holes drilled to accommodate EPR tubes, were used (required in particular for pulsed EPR studies). In this way the difficulty in transferring a solid mitochondrial membrane mass into narrow EPR tubes was overcome. The tubes were then centrifuged at $27000 \times g$ for 15 min. For ESEEM studies, EPR tubes with ≤ 4 mm diameter were chosen so as to pack as much material into the bottom ~ 15 mm of the EPR tube; the upper limit being set by the dimensions of the cavity. The membranes were then selectively reduced for the Rieske [2Fe-2S] cluster of Complex III with 2 mM ascorbate and 100 μM TMPD for 10 min, under an argon atmosphere. Some of the membrane preparations were treated with 0.1 mM UHDBT (in dimethylsulphoxide) for 10 min, prior to reduction. UHDBT was a gift from Dr A. Chapman, Wellcome Laboratories, Beckenham, Kent.

EPR measurements

X-band c.w.-EPR spectra were recorded on a Bruker ESP300 spectrometer with a TE102 rectangular cavity, and an Oxford Instruments ESR900 liquid helium flow cryostat. ENDOR spectra were recorded with the Bruker multiple resonance accessory, with a TM110 cavity and an A-500 RF power amplifier. Pulsed EPR measurements were recorded at X-band on a Bruker ESP380 spectrometer, with a dielectric variable-Q resonator in an Oxford Instruments CF 935 liquid helium cryostat. The ESEEM data were collected and Fourier transformed as described elsewhere [9].

3. Results and discussion

c.w. EPR spectral features of the bovine heart Rieske centre

The highly concentrated bovine heart membranes were progressively reduced by the addition of 2 mM ascorbate and 100 μM TMPD in quartz tubes, and the state of the mitochondrial iron-sulphur clusters monitored by EPR spectroscopy of frozen samples. Treatment with ascorbate and TMPD for 10 min led to preferential reduction of the Rieske [2Fe-2S] cluster. The rhombic c.w. EPR spectrum (Fig. 1a) observed at 18 K with the reduced Rieske cluster shows features at $g_{yx} = 1.890$ and 1.796. The trough at $g_x = 1.796$ is very broad and shallow, and spans ~ 25.0 mT. In the 8–21 K temperature range, the g_z feature of the Rieske cluster is difficult to see, due to overlap from the $g = 2.01$ signal from the oxidised [3Fe-4S] cluster of Complex II Centre S3. This suggests that the redox potential obtained upon treating the membranes with ascorbate and TMPD was higher than, or comparable with, that of the [3Fe-4S] cluster, $\geq +60$ mV. A minor reduction of Complex II Centre S1 ($E_m = 0$ mV) is also apparent, as a small signal characteristic of its [2Fe-2S] cluster is detectable at $g = 1.94$ (Fig. 1a).

On lowering the redox potential further, to below + 60 mV, in an attempt to reduce the [3Fe-4S] cluster and hence make it EPR-silent, an EPR spectrum exhibiting a signal solely from the Rieske [2Fe-2S] cluster was not observed. Instead, the $g = 1.94$ signal of Centre S1 tended to dominate the spectrum, as a redox potential of 0 mV was reached. In addition, at higher temperatures (30–60 K), the membranes showed a free

radical signal at $g = 2.001$ (linewidth ~ 3.3 mT), superimposed on the g_z-signal of the Rieske [2Fe-2S] cluster.

Upon treatment of the membranes with the Q_0-site inhibitor UHDBT, a shift in the EPR g_{zyx} factors of the Rieske [2Fe-2S] cluster to 2.027, 1.890 and 1.770 was observed (Fig. 1b). The g_x-trough became deeper and spanned only ~ 21 mT, as observed previously [35]. Once again, at low temperatures, the spectrum exhibited a signal typical of Centre S3, and at 60 K, a free radical signal at $g = 2.00$.

ENDOR spectral features of the bovine heart rieske centre

X-band ENDOR measurements were performed on the ascorbate- and TMPD-reduced Rieske [2Fe-2S] cluster in bovine heart membranes. Although the proton Larmor frequency would be at its maximum with the magnetic field set on the $g_x = 1.80$ resonance, and thus slightly less likely to interfere with the ^{14}N signals in the ENDOR spectrum, the data presented here were recorded at $g_y = 1.89$ for the best signal-to-noise ratio.

The $g_y = 1.89$ ^1H ENDOR spectrum (Fig. 2A) clearly displays a broad feature centred at $\nu_H = 15.15$ MHz, with resonances to both low- and high-frequency of ν_H, due to coupled protons. There appear to be about eight resolved pairs of proton resonances in the 13–17 MHz region (as indicated by the stick diagram in the figure), arranged symmetrically around ν_H. As we can detect pairs of resonances centred about ν_H in the 10–20 MHz region of the ENDOR spectra, these features are unlikely to be due to nitrogen.

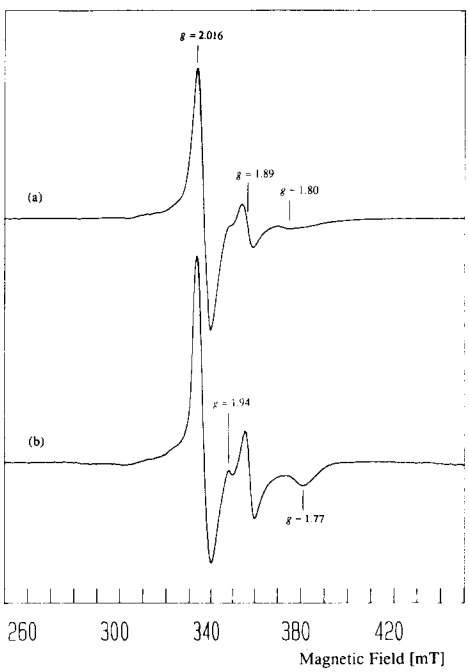

Fig. 1. X-band c.w. EPR spectra of ascorbate- and TMPD-reduced bovine heart mitochondrial membranes. The spectra were recorded at 18 K with membranes that were either in the (a) control, or (b) UHDBT-pretreated state, prior to reduction. ENDOR and pulsed EPR studies were subsequently conducted on these samples. Measurement conditions were: microwave power, 20 mW; microwave frequency, (a) 9.4327, (b) 9.4342 GHz; modulation amplitude, 1.0 mT; time constant, 0.328 s; sweep rate, 4.769 mT/s. The spectra are an average of 3 scans.

Given the probable presence of two histidine ligands [12], candidates for the protons with the stronger couplings of $A_H \sim 3.0$–7.0 MHz are those on the carbons of the imidazole ring closest to [Fe^{2+}-^{14}N] or the β-protons of the two coordinating cysteines [Fe^{3+}-^{32}S-CH_2-]. Candidates for the more weakly-coupled protons could be those on the carbons located further away from the [Fe^{2+}-^{14}N] or [Fe^{3+}-^{32}S-CH_2-] bonds, or possibly ubiquinol. Various theories have proposed a molecule of ubiquinone or ubiquinol to be coordinated to one or both of the proposed histidine ligands of the Rieske cluster [25,33]. We have observed no significant change in the ^1H ENDOR spectrum on lowering the pH of the membranes from 7.5, to 6.5 and 6.0.

The low-frequency $g_y = 1.89$ ENDOR spectrum (Fig. 2B) displays features in the 3–8 MHz region. A number of peaks are detectable in this region of the spectrum, including a number of doublets, all of which show a splitting of $2\nu(^{14}N) \sim 2.2$ MHz. These resonances do not have the high-frequency ν_+ partners required for protons. We therefore assign the features to ^{14}N-nuclei. In principle, the first-order ENDOR spectrum of a ^{14}N nucleus displays a four-line pattern, as the Larmor-split doublet is further split by the quadrupolar term. In the case of a metal centre coordinated to histidine, a simpler two-line pattern (a single Larmor-split doublet) centred at $A^N/2$ is obtained, as the quadrupole interaction is often small and unresolvable [36]. Furthermore, at an intermediate g-factor position, the ENDOR spectrum observed is not due to a single orientation, although it does arise from a well-defined subset of molecular orientations [37,38]. As the spectrum may be further complicated by hyperfine anisotropy, we have made no detailed analysis of the low-frequency features.

An investigation of the nature of the ligands to the bovine heart Rieske iron-sulphur cluster by ESEEM spectroscopy

The ESEEM technique was applied to the Rieske cluster in the same samples of intact mitochondrial membranes. The ESEEM data presented below were obtained by the three-pulse (90°-τ-90°-T-90°) or 'stimulated echo' sequence. The experiments were performed at various values of B_0 corresponding to the principal g-factors of the Rieske [2Fe-2S] cluster.

Fig. 3A shows the three-pulse ESEEM spectrum obtained at $g_y = 1.89$, using a τ-value of 112 ns, calcu-

lated for suppression of modulations due to weakly coupled protons. The presence of a matrix proton line at ~ 15.5 MHz (i.e., from the remote proton nuclei in the protein or water environment), indicates that the τ-value used has only partially suppressed the weakly coupled protons. As τ is limited to steps of 8 ns on our instrument it is not possible to exactly match τ to the proton Larmor frequency, and hence totally suppress the matrix protons.

At least six lines are clearly resolved in the 0–10 MHz region of the Fourier transform spectrum, at ~ 1.1, 2.40, 3.96, 5.1, 6.20 and 7.88 MHz (Fig. 3B). These modulation frequencies are consistent with strong coupling to ^{14}N, and are strong evidence that the spectra observed in these samples of intact membranes are specifically due to the Rieske cluster. The modulation frequencies are similar to those observed in isolated Rieske-type proteins, and in purified respiratory-chain complexes [10–12], which have been assigned to two directly-coupled nitrogen ligands. The modulation frequencies extend to higher values than those observed with other [2Fe-2S] clusters such as plant-type ferredoxin, and Complex II Centre S1 [7,39], which have been assigned to indirectly-coupled peptide nitrogens.

Interpretation of the modulation frequencies

We have applied the theoretical and graphical analyses of Dikanov et al. [40,41], in order to analyse the ESEEM frequencies observed with the Rieske [2Fe-2S] cluster, as described previously [42]. A full simulation of an ESEEM spectrum, with couplings to nitrogens, involves the diagonalisation of a hamiltonian including the electronic Zeeman, nuclear hyperfine, nuclear Zeeman and quadrupole interactions. The 'powder' spectrum of a frozen solution is then obtained by averaging the spectrum for all molecular orientations relative to the applied magnetic field. A simpler method of analysis is applicable when the hyperfine interaction is significantly greater than the nuclear Zeeman term [40,41]. Then, for many molecular orientations, the so-called double-quantum ($\Delta m_i = 2$) transitions are relatively constant, whereas the single-quantum ($\Delta m_i = 1$) transitions are more variable. Therefore the double-quantum

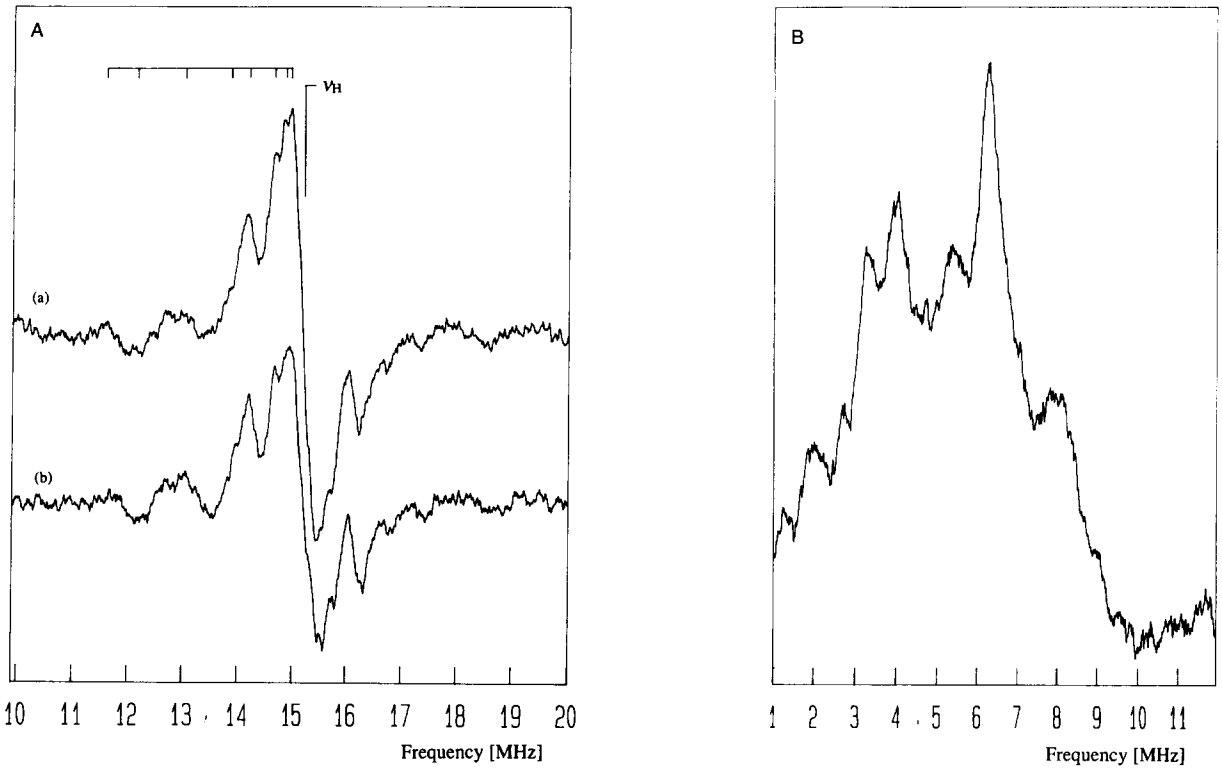

Fig. 2. (A) X-band ^1H ENDOR spectra of the ascorbate- and TMPD-reduced Rieske cluster in bovine heart mitochondrial membranes, at $g_y = 1.89$. Measurement conditions were: temperature, 8 K; microwave power, 3.99 mW; radiofrequency phase, 30°; modulation depth, 158.11 kHz; microwave frequency, (a) 9.4408, (b) 9.4452 GHz; B_o, (a) 356.57, (b) 355.65 mT; sweep rate, 0.1431 MHz/s. Each spectrum is an average of 50 scans. (B) X-band ^{14}N ENDOR spectrum of the ascorbate- and TMPD-reduced Rieske cluster in bovine heart mitochondrial membranes, at $g_y = 1.89$. Measurement conditions were as for Fig. 2A, except: microwave frequency, 9.4394 GHz; B_o, 355.74 mT; sweep rate, 0.1311 MHz/s. The spectrum has had the cavity background signal subtracted, and represents an average of 190 scans.

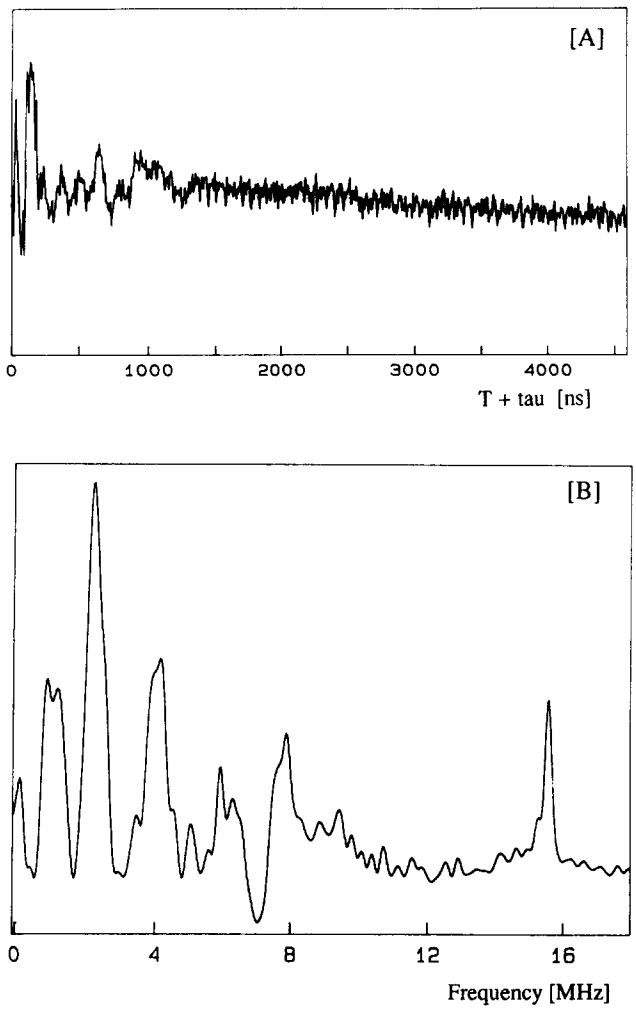

Fig. 3. Three-pulse ESEEM spectra of the ascorbate- and TMPD-reduced bovine heart Rieske protein in control membranes at $g_y = 1.89$. (A) echo decay envelope, and (B) Fourier transform. Measurement conditions were: temperature, 3.7 K; τ-value, 112 ns; pulse width (for a 90° pulse), 16 ns; bandwidth, 100 MHz; microwave frequency, 9.7201 GHz; B_o, 367.61 mT; number of shots, 30; video amplifier gain, 60 dB; travelling wave tube attenuation, 0 dB; shot repetition time, 30.7 ms.

($\Delta m_i = 2$) transitions lead to prominent features in the frequency-domain spectrum. The frequencies $\nu_{dq}^{\pm}$ are given by:

$$\nu_{dq}^{\pm} = 2\left[\nu_{ef}^2 + K^2(3+\eta^2)\right]^{1/2} \quad (1)$$

where

$$\nu_{ef} = |\nu_i \pm |A|/2| \quad (2)$$

and

$$K^2 = (e^2qQ/4) \quad (3)$$

ν_i is the nuclear Zeeman frequency at the observing magnetic field, η is the asymmetry parameter, and A and e^2qQ are the hyperfine and quadrupolar coupling parameters, respectively. The value of η cannot be estimated without a full spectrum simulation, but values over the possible range (0 to 1) make only a minor uncertainty (about $\pm 15\%$) in the estimates of A and e^2qQ.

For two nitrogen nuclei, we expect two pairs of double-quantum frequencies, (ν_{dq}^- and ν_{dq}^+). In order to estimate the hyperfine and quadrupole parameters we need to assign the pairs which belong to each nucleus. Following Britt et al. [12], we calculated the expected values of A and e^2qQ, assuming the two different possible pairings of higher and lower frequencies. For example in Fig. 3B, prominent modulation frequencies are observed at 2.40, 3.96, 6.20 and 7.88 MHz. Suppose we assume that the lines at 2.40 and 6.20 MHz correspond to the ν_{dq}^- and ν_{dq}^+ transitions of one ^{14}N ligand, and the lines at 3.96 and 7.88 MHz to the second; using Eq. (1) the isotropic hyperfine couplings for the two nitrogens are $A_1 = 3.6$ MHz and $A_2 = 5.2$ MHz and the quadrupolar couplings are $e^2qQ_1 = 2.1$–2.4 and $e^2qQ_2 = 2.7$–3.15 MHz (see Table 1). However, the alternative pairing arrangement gives $e^2qQ_1 = 0$ and $e^2qQ_2 = 4.0$–4.6 MHz. As these latter values are unlikely for a ^{14}N nucleus in the protein environment, this choice is considered to be incorrect. For example, for the imino nitrogen of the imidazole ring, coordinated to the metals zinc and cadmium, e^2qQ values of 1.92–2.82 MHz (for $\eta = 0.69$–0.25) have been determined [43].

Table 2 compares our estimates of the hyperfine and quadrupole couplings for the ^{14}N nuclei coordinated to the Rieske [2Fe-2S] cluster, with those of other proteins in the literature. The couplings are similar, but not identical, to those obtained with the purified bc_1 complex, chloroplast b_6f complex, and *P. cepacia* PDO [12,27,28]. The small differences may

Table 1
Hyperfine (A) and quadrupolar (ξ, e^2qQ) coupling values for the bovine heart Rieske cluster in control and UHDBT-treated mitochondrial membranes, determined at g_y

$\Delta m_i = 2$ Transition	Features in the Fourier transform (MHz)		Principal values (MHz)			
			A [a]		e^2qQ [b]	
	N_1	N_2	N_1	N_2	N_1	N_2
Control Rieske cluster						
ν_{dq}^-	2.40	3.96				
			3.55	5.20	2.1–2.4	2.7–3.15
ν_{dq}^+	6.20	7.88				
UHDBT-treated Rieske cluster						
ν_{dq}^-	2.56	4.00				
			3.36	5.34	2.3–2.65	2.6–3.0
ν_{dq}^+	6.07	8.01				

[a] The hyperfine coupling constant was determined under the assumption that the coupling is isotropic (i.e., $A_x = A_y = A_z$).
[b] A range of values were determined for the quadrupolar coupling constant e^2qQ by varying the asymmetry parameter (η) between 0 and 1.

indicate slight differences in coordination geometry. However, the differences may not be significant in view of the fact that our simple analysis of the ESEEM data has not taken account of hyperfine anisotropy, which may explain the discrepancies observed between the data.

So far our interpretation of the $g_y = 1.89$ ESEEM spectrum of the bovine Rieske cluster has been restricted to the four highest prominent modulation frequencies. However, other peaks are clearly present in the Fourier transform spectrum and these still have to be assigned. These transition frequencies could be due to:

(i) the 'single-quantum' transitions for each of the two imidazole ^{14}N ligands, or
(ii) a weak interaction of the Rieske [2Fe-2S] cluster with a peptide nitrogen, as is observed with [2Fe-2S] clusters coordinated by four cysteine residues [7,8,39].
(iii) combination lines from interactions with multiple nuclei.

Effects of the quinone analogue UHDBT

ESEEM and ENDOR spectra were recorded of the Rieske cluster in SMP treated with UHDBT. The ENDOR spectra, recorded at g_y and g_z, showed that the ^1H couplings were very similar to those observed with the native Rieske cluster (Fig. 2A). Moreover, ESEEM spectra, recorded at $g = 1.89$ (Fig. 4), showed very similar ^{14}N coupling parameters to the native Rieske cluster (Table 1). These results indicate that no significant change in the coordination geometry of the [2Fe-2S] cluster occurs on treatment with the quinone analogue. The changes in the g-factors on treatment with UHDBT (Fig. 1) probably reflect subtle changes in coordination geometry.

It has been shown here, for the first time, that the ESEEM technique can be used to detect the nitrogen ligands of the Rieske [2Fe-2S] cluster in intact mitochondrial membranes. Our data have provided supporting evidence that two ^{14}N ligands coordinate the native and UHDBT-treated Rieske [2Fe-2S] cluster in its natural mitochondrial membrane environment. The other two ligands are presumed to be cysteine sulphurs. This is in contrast to the classical ferredoxin-type

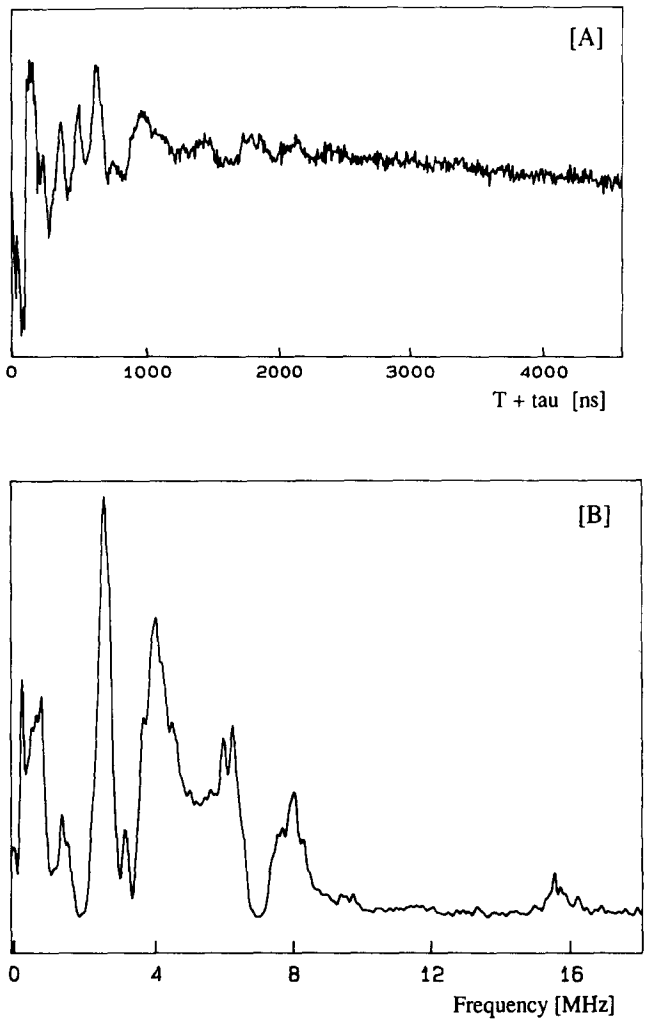

Fig. 4. Three-pulse ESEEM spectra of the UHDBT-treated bovine heart Rieske protein in membranes, reduced with ascorbate and TMPD, at $g_y = 1.89$. (A) echo decay envelope, and (B) Fourier Transform. Measurement conditions were as for Fig. 3, except: microwave frequency, 9.7052 GHz; B_0, 367.61 mT; number of shots, 45.

Table 2
Summary of ESEEM and ENDOR superhyperfine frequencies of various Rieske and Rieske-type proteins

Protein	Method	A (MHz)		e^2qQ (MHz)		Ref.
		N_1	N_2	N_1	N_2	
P. cepacia PDO	ENDOR[1]	4.3	5.5	2.3	2.6	[27]
R. capsulatus cyt c_2 oxidoreducase	ENDOR[2]	4.5	5.5	–	–	[28]
Spinach Cyt b_6f complex	ESEEM[3]	3.8	4.6	2.5–2.9	2.5–2.9	[12]
Cyt bc_1 complex, in bovine heart mitochondrial membranes	ESEEM[4]	3.6	5.2	2.1–2.4	2.7–3.15	this work
UHDBT-treated Cyt bc_1 complex, in bovine heart mitochondrial membranes	ESEEM[5]	3.4	5.3	2.3–2.65	2.6–3.0	this work

The g-factors were: (1,3) 1.92; (2) 1.90; (4,5) 1.89.

[2Fe-2S] clusters in which all coordination is by cysteinyl sulphur ligands. The amino acid sequence of the bovine heart Rieske protein has been determined, and contains four cysteines (C139, 144, 158 and 160) and only two histidines (H141 and 161), in homologous positions to conserved residues in other Rieske-type proteins [44]. The consensus sequence which has been suggested to bind the cluster [45] is:

C-X-H- 15 to 17 amino acids -C-X-X-H

On the basis of sequence conservation, and site-directed mutagenesis studies of the *R. capsulatus* protein [46], the ligands to the bovine heart Rieske cluster are suggested to be provided by histidine residues 141 and 161, and cysteinyl residues 139 and 158.

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5. References

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