Volume 214, number 1, 117–121 FEB 04570 April 1987

Evidence for N coordination to Fe in the [2Fe-2S] center in yeast mitochondrial complex III

Comparison with similar findings for analogous bacterial [2Fe-2S] proteins

Joshua Telser*, Brian M. Hoffman*, Russell LoBrutto+, Tomoko Ohnishi+, Ah-Lim Tsai, David Simpkin and Graham Palmer

Department of Chemistry, Northwestern University, Evanston, IL 60201, +Department of Biochemistry & Biophysics, University of Pennsylvania, Philadelphia, PA 19104 and Department of Biochemistry, Rice University, Houston, TX 77251, USA

Received 14 January 1987

Yeast mitochondrial complex III contains a subunit with a [2Fe-2S] cluster (the Rieske center) that has unusual physical and chemical properties. For apparently similar centers isolated from bacteria, it has been shown by electron nuclear double resonance (ENDOR) and electron spin echo envelope modulation (ESEEM) measurements that these [2Fe-2S] centers are coordinated by at least one and probably two nitrogen ligands. This work describes similar ENDOR and ESEEM studies on the intact mitochondrial complex. We find that this [2Fe-2S] cluster exhibits ESEEM and ENDOR properties that appear to be indistinguishable from those observed with the isolated bacterial systems. Furthermore, changes in EPR lineshape that occur as complex III is progressively reduced are not accompanied by any changes in the nitrogen coupling parameters. This spectroscopic evidence for nitrogen coordination is supported by published sequence data on four Rieske iron-sulfur subunits. It seems likely that this is a general characteristic of such [2Fe-2S] redox active centers.

Mitochondria; Complex III; Iron-sulfur cluster; ENDOR; ESEEM

1. INTRODUCTION

Ubiquinol-cytochrome *c* oxidoreductase (complex III) isolated from the mitochondrial inner membrane contains 2 *b* cytochromes, one *c*-type cytochrome (c_1), one [2Fe-2S] cluster (the Rieske center) and ubiquinone (Q_6 in yeast) at approximately equivalent concentration to cytochrome c_1. The [2Fe-2S] cluster is unusual in that: (i) its midpoint redox potential of about 280 mV in both bovine heart and yeast is atypically high compared with values of -200 to -400 mV observed with more typical [2Fe-2S] clusters [1]: (ii) the high-field EPR *g* value (g_x) is quite small, ~ 1.8 [1]; and (iii) the cluster does not appear to have the intense visible CD usually associated with a [2Fe-2S] cluster (Tsai, A.-L. and Palmer, G., unpublished).

Proteins with similar characteristics exist in bacteria, notably the phthalate dioxygenase from *Pseudomonas cepacia* and the Rieske iron-sulfur protein from *Thermus thermophilus*. Recently, Mossbauer spectroscopic measurements indicated that the Fe ions of the clusters in these proteins are not coordinated solely by cysteine mercaptide ligands [2]. Electron nuclear double resonance (ENDOR) demonstrated that one or, more likely, two different types of nitrogen atoms are directly coordinated to the iron-sulfur cluster [3]. A strong

Correspondence address: J. Telser, Dept of Chemistry, Northwestern University, Evanston, IL 60201, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

signal observed at 11–14 MHz was assigned as resulting from one coordinated nitrogen (or a set of equivalent nitrogen ligands) with a coupling constant of A_N 26–28 MHz. A weaker feature at ~ 4.5 MHz was assigned as a second nitrogen coordinated to a metal ion because its coupling, $A_N \sim 9$ MHz, is larger than those previously assigned to the remote nitrogen of histidine. The stronger coupling was too large to be detectable by electron spin echo envelope modulation (ESEEM) spectroscopy, but modulation frequencies between 5 and 7 MHz in the ESEEM spectrum confirmed the second coupling. The spectrum also contained lower frequencies (0.8–4.8 MHz), some or all of which could be due to the ^{14}N coupling from the remote nitrogen of histidine [3,4].

In view of the importance of this finding, we felt it was necessary to establish whether nitrogen coordination was also involved in the cluster integrated into mitochondrial complex III. Furthermore, the EPR lineshape of the iron-sulfur cluster varies with the degree of reduction of the complex. When only the cluster and cytochrome c_1 are reduced (e.g. by addition of the weak reductant, ascorbate), the $g_x = 1.81$ EPR feature has a relatively narrow width. Upon reduction of all of the components (e.g. by addition of dithionite), g_x changes to 1.79 and the EPR linewidth at this feature increases markedly [5]. This transition appears to be associated with the reduction of Q_6. It was therefore of interest to learn whether this change in lineshape was associated with any change in coordination.

We find that the [2Fe-2S] cluster of mitochondrial complex III indeed exhibits N-coordination, and that this coordination is spectroscopically indistinguishable from that in the purified bacterial proteins. In addition, changes in the EPR lineshape that occur as complex III is progressively reduced do not lead to any discernible change in the nitrogen hyperfine coupling pattern, and thus do not arise from a change in the nitrogenous ligands to the cluster.

2. PROCEDURES AND THEORY

Complex III was isolated from yeast mitochondrial fragments by the method of Siedow et al. [5] and concentrated to about 450 μM with a Micro-ProDicon concentrator (Bio-Molecular Dynamics). Portions of this enzyme solution were placed in two EPR tubes. A small excess of solid sodium ascorbate (Sigma) was added to one EPR tube and excess solid sodium dithionite (Virginia Smelting) was added to the second. The samples were frozen and EPR spectra recorded at 12 K verified that each tube contained the expected spectral species [5]. Samples for ESE measurements were obtained by the controlled addition of reductant to enzyme pretreated with funiculosin. This inhibitor raises the redox mid-point potential of the iron-sulfur cluster by 150 mV thus allowing this component to be reduced while all other centers are still oxidized [6].

ENDOR spectra were recorded on a modified Varian E109 spectrometer described in [7]. The first-order ENDOR spectrum of a set of magnetically equivalent ^{14}N nuclei consists, in principle, of four transitions at frequencies given by eqn 1 [8,9].

$$\nu^N_\pm = A^N/2 \pm \nu_N \pm 3P^N/2 \quad (1)$$

where A is the hyperfine interaction constant, P is the quadrupole coupling constant, and ν_N is the nuclear Larmor frequency. Eqn 1 describes a four-line pattern centered at $A/2$, provided $A/2 \gg \nu_N, P$ as is true here. Normally, in biological metal centers involving coordinated histidine, the quadrapole coupling is small and not resolved, giving a two-line pattern centered at $A/2$ [10]. A set of magnetically equivalent protons is expected to give a single pair of ENDOR transitions separated by A^H and centered about the free-proton Larmor frequency, ν_H ($= g_H\beta_n H_0/h$; 16.18 MHz at 0.380 T):

$$\nu^H_\pm = | \nu_H \pm A^H/2 | \quad (2)$$

Measurements of ESEEM spectra were made with a low-power (38 W) electron spin echo (ESE) spectrometer as in [11]. A custom-built microwave resonator, consisting of a copper-foil strip mounted on the outside of the quartz sample tube, was used for this work [11]. A 'stimulated echo' pulse sequence ([90°-τ-90°-T-90°-τ-echo], where τ is fixed and T is scanned from 0 to 10 μs) was employed for ESEEM measurements. This sequence produces a spin echo which, in the presence of weak nuclear superhyperfine couplings, has sinusoidal modulations superimposed upon its decay envelope as T is scanned [12]. The frequencies of the modulations can reveal both the iden-

tities of coupled nuclei, and, in some cases, the magnitudes of the couplings (as in ENDOR). The method is complementary to ENDOR in that ENDOR is most successful at measuring strong couplings, while only weakly coupled nuclei will produce the ESEEM effect.

The frequency components of the ESEEM spectrum were determined by first fitting the spin echo envelope to an exponential function, subtracting this function from the data, and then computing the Fourier transform of the residual modulations. The transformed (frequency domain) spectrum is presented in squared form, since instrumental dead time following the pulses precluded the computation of a properly phased spectrum.

3. RESULTS

ENDOR measurements on both purified phthalate dioxygenase from *P. cepacia* and *T. thermophilus* Rieske iron-sulfur protein showed two ENDOR signals attributed to coordination of the [2Fe-2S] cluster by nitrogenous ligands [3]. Samples of the Rieske center from intact yeast mitochondrial complex III have been studied in an analogous manner. Fig. 1 presents the higher frequency range of the ENDOR spectrum of both the ascorbate (panel A) and dithionite-reduced (panel B) samples. In the former, only the [2Fe-2S] cluster and cytochrome c_1 are reduced. These spectra were obtained by setting the magnetic field to the high-field edge of the EPR signal, at $g_x = 1.79$. Close examination of the ENDOR signals from the two states of the protein shows no meaningful difference. At this magnetic field, the proton pattern is at the highest frequency (eqn 2) and interferes least with the ^{14}N signals. In addition to the strong pattern symmetric about ν_H (16.18 MHz at 0.380 *T*) arising from ^1H resonances, another signal was observed at the low-frequency side of the proton pattern and must arise from a nitrogen with A^N = 26–28 MHz that is coordinated to one of the iron ion(s) of the Rieske center in complex III. Since the ENDOR spectra for the two states of complex III are the same, it is clear that the unusual coordination by nitrogen is unaffected by the choice of reductant. Furthermore, there is no meaningful difference between these spectra and those of the isolated bacterial proteins [3].

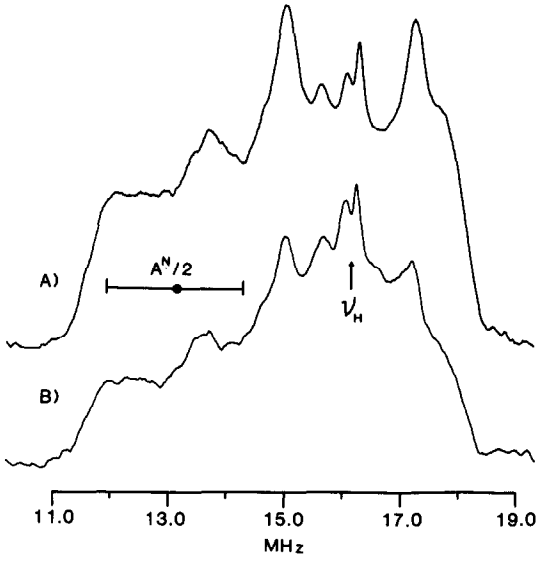

Fig. 1. High-frequency ENDOR spectra of ascorbate (A) and dithionite (B) reduced Rieske iron-sulfur center in mitochondrial complex III. Conditions: (A) ~0.45 mM in 0.1 M KP_i, pH 7.4, 1 mM EDTA, 0.1% deoxycholate, 0.1% Triton QS30; dispersion mode EPR; temperature, 2 K; microwave frequency, 9.535 GHz; microwave power, 20 μW; magnetic field, 0.3800 T ($g = 1.79$); time constant, 0.064 s; modulation amplitude, 0.10 mT; modulation frequency, 100 kHz; RF scan rate, 1 MHz/s; 925 scans. (B) As in A except: time constant 0.128 s; modulation amplitude, 0.05 mT; RF scan rate, 0.5 MHz/s; 640 scans. (●) $A^N/2$, (|—|) $2\nu_N$.

In addition to the strongly coupled nitrogen(s) described above, a weaker signal is observed at lower frequency as was also found earlier with the bacterial Rieske proteins. Fig. 2 presents the low-frequency ENDOR spectrum of both ascorbate (panel A) and dithionite-reduced (panel B) complex III samples. Both samples show a feature at 4–5 MHz, and again there is no discernible difference between the spectrum obtained with the two reductants. The possibility exists that this weak signal arises from the remote nitrogen of a histidine imidazole coordinated to the cluster and the strong signal from the directly coordinated nitrogen. However, the observed pattern corresponds to A_N ~9 MHz, which seems to be too large for coupling to the distal nitrogen of a coordinated histidine. It is also too large for a dipolar interaction with a non-coordinated nitrogen. It thus seems most plausible that this second ENDOR

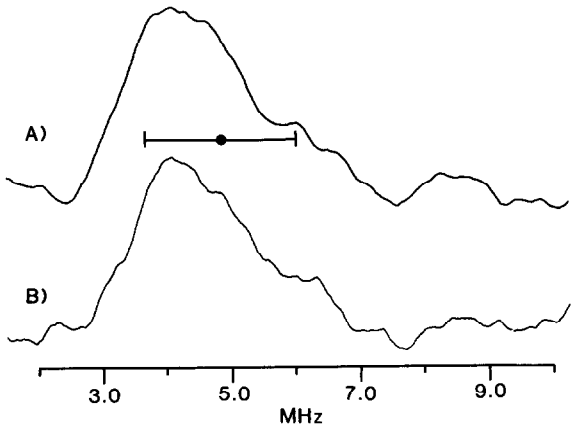

Fig. 2. Low-frequency ENDOR spectra of ascorbate (A) and dithionite (B) reduced Rieske iron-sulfur center in mitochondrial complex III. Conditions: (A) as in fig. 1A except: time constant, 0.032 s; modulation amplitude, 0.40 mT; RF scan rate, 5 MHz/s, 650 scans. (B) As in A except: 400 scans. The baseline in these spectra has been corrected by computer subtraction of a curve generated by a least-squares fit of the background to a second-order polynomial. Symbols as in fig. 1.

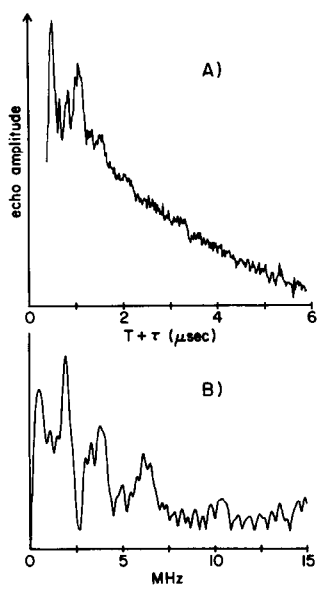

Fig. 3. Time-domain (A) and frequency domain (B) ESEEM spectra of the Rieske iron-sulfur cluster in yeast mitochondrial complex III. Data were obtained at 7.5 K, from a sample with all three cytochromes in the ferric (paramagnetic) state. Other conditions: $H_0 = 0.3383$ T; microwave frequency = 9028 MHz; $\tau = 278$ ns; spectrum is an average over 32 30-s sweeps, at a pulse sequence repetition rate of 333 Hz.

feature arises from coordination to a second species of nitrogen.

The results of the three-pulse ESEEM experiment are shown in fig. 3. In addition to frequencies between 0 and 5 MHz, which are commonly observed in [2Fe-2S] proteins [4] and are presumed to arise from coupling with polypeptide backbone nitrogen (multiple NH...S hydrogen bonds have been observed in the X-ray structure [13] of iron-sulfur proteins), there is a broad peak centered at about 6.3 MHz. Comparison with fig. 2 reveals that this peak is most likely due to the same 9 MHz coupling as produced the lower-frequency ENDOR resonance. The ESEEM spectrum in fig. 3 was obtained from a sample poised such that the iron-sulfur cluster was reduced, but all three cytochromes (b_H, b_L, c_1) were oxidized. Virtually identical results were obtained as each of the cytochromes was reduced in sequence. This supports the observation by ENDOR that the ^{14}N couplings are independent of the overall redox state of the complex.

Very recently it has been reported that ENDOR measurements on the cytochrome b_6f complex of spinach chloroplasts did not reveal any features that could be assigned to a nitrogen nucleus though contributions from protons were readily detected [14]. In view of the marked structural homologies between cytochrome b_6f and the mitochondrial complex III (bc_1) [15], it seems certain that the reported failure to observe the nitrogen ENDOR in the plant enzyme complex reflects the use of instrumental conditions unsuitable for visualizing nitrogen, for the ENDOR signal of this species is not necessarily maximal under conditions which maximize the contributions of protons. Indeed, a careful examination of the published ENDOR spectrum suggests that the intensities are not symmetric about ν_H and may in fact contain contributions from ^{14}N resonances.

The phase memory time, T_M, of the iron-sulfur cluster in funiculosin-treated enzyme in which the cytochromes were either fully oxidized or fully reduced was measured using the spin-echo method. It was found that conversion of all three hemes to the diamagnetic, ferrous state led only to a small ($\sim$2-fold increase in T_M. This implies that dipolar interactions between the hemes and the iron-sulfur cluster are small, and that no heme is within $\sim$10 Å of the iron-sulfur cluster.

4. DISCUSSION

The spectroscopic correspondence of ^{14}N ligation to the Rieske centers in isolate *Thermus* and *Pseudomonas* proteins with the center in intact yeast mitochondrial complex III demonstrates the generality of this unusual structural feature of nitrogen ligation to an iron-sulfur center. This result is consistent with the early prediction of Blumberg and Peisach [17] that an unusually large range of g values corresponds to a change in chemical composition at or near the [2Fe-2S] cluster. This coordination environment may be reponsible for the high redox midpoint potential of the Rieske cluster compared to [2Fe-2S] ferredoxin systems by reducing the net charge on the cluster from $-2/-3$ to $0/-1$ and by coordinating the Fe atoms(s) with more electronegative atoms, both effects making the Rieske center a better electron acceptor. In addition, it is significant that the ^{14}N signals in the two complex III samples, prepared using different reductants, are virtually identical. The EPR spectra of ascorbate and dithionite reduced protein show a clear difference in the g_x value of and in the associated linewidth. However, because this effect is not manifested in the ^{14}N ENDOR signals, the nitrogen ligation appears to be the same in both samples.

The sequence of the iron-sulfur subunit has been obtained from yeast [16], *Neurospora crassa* [18], *Rhodopseudomonas sphaeroides* [19], and the spinach cytochrome b_6f complex [20]. Only four cysteines are conserved in a strongly homologous stretch of about 50 residues; these cysteines are not arranged in the characteristic three plus one arrangement found with, e.g. spinach ferredoxin. Rather, they are arranged as two pairs with each having an adjacent histidine, viz. Cys-X-His-Leu-Gly-Cys-13 (X)-Cys-Pro-Cys-His. This unusual sequence of residues supports the proposition [3] that histidine is a ligand to iron in this redox center.

ACKNOWLEDGEMENTS

This work was supported by National Intitutes of Health grants HL 13531 (B.M.H.), GM 23371 (G.P.), and GM 25052 (to J. Leigh), the Robert E. Welch Foundation C-636 (G.P.), National Science Foundation grant DMB 8606575 (B.M.H.), and NRSA award GM 10604-02 (J.T.).

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