The Interaction of the Rieske Iron-Sulfur Protein with Occupants of the Q<sub>c</sub>-site of the bc<sub>1</sub> Complex, Probed by Electron Spin Echo Envelope Modulation*

Received for publication, November 19, 2001, and in revised form, December 13, 2001
Published, JBC Papers in Press, December 17, 2001, DOI 10.1074/jbc.C100664200

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The bifurcated reaction at the Q<sub>c</sub>-site of the bc<sub>1</sub> complex provides the mechanistic basis of the proton pumping activity through which the complex conserves redox energy in the proton gradient. Structural information about the binding of quinone at the site is lacking, because the site is vacant in crystals of the native complexes. We now report the first structural characterization of the interaction of the native quinone occupant with the Rieske iron-sulfur protein in the bc<sub>1</sub> complex of *Rhodobacter sphaeroides*, using high resolution EPR. We have compared the binding configuration in the presence of quinone with the known structures for the complex with stigmatellin and myxothiazol. We have shown by using EPR and orientation-selective electron spin echo envelope modulation (ESEEM) measurements of the iron-sulfur protein that when quinone is present in the site, the isotropic hyperfine constant of one of the N<sub>2</sub> atoms of a liganding histidine of the [2Fe-2S] cluster is similar to that observed when stigmatellin is present and different from the configuration in the presence of myxothiazol. The spectra also show complementary differences in nitrogen quadrupole splittings in some orientations. We suggest that the EPR characteristics, the ESEEM spectra, and the hyperfine couplings reflect a similar interaction between the iron-sulfur protein and the quinone or stigmatellin and that the N<sub>2</sub> involved is that of a histidine (equivalent to His-161 in the chicken mitochondrial complex) that forms both a ligand to the cluster and a hydrogen bond with a carbonyl oxygen atom of the Q<sub>c</sub>-site occupant.

The cytochrome (cyt)<sup>1</sup> bc<sub>1</sub> complex family plays an essential role in the energy metabolism of the biosphere (1, 2), providing the central enzymes in the electron transfer chains of all the major pathways. In bacteria, and their mitochondrial and chloroplasts descendants, the complex catalyzes oxidation of quinol and reduction of a diffusible small protein (cyt<sub>c</sub> or cyt<sub>c<sub>2</sub></sub>) in α-proteobacteria) through a modified Q-cycle (3–6).

Interest in the bc<sub>1</sub> complex has been heightened by the recent availability of structures and the new insights into functional aspects that these have encouraged (2, 7–12). However, with the structural ensemble come the realization that the static models do not provide all the information needed to understand mechanism at a deeper level. The reactions of greatest interest are the proton-coupled electron transfers at the quinone-processing sites. In particular, oxidation of ubiquinol at the Q<sub>c</sub>-site of the complex is the key reaction of the Q-cycle, providing the bifurcation of electron transfer between two different acceptor chains that allows coupling to proton transfer. The Q<sub>c</sub>-site of the mitochondrial complex, and its mechanism, are also of central importance to the physiology of cellular damage and aging, because it is a major site for generation of reactive oxygen species.

To understand the mechanism, we need detailed information about the local reaction environment, including protein structure, hydrogen bonding, and distances, to provide the parameters that control rates, and partitioning of electrons to different pathways. Of the five well characterized paramagnetic centers of the bc<sub>1</sub> complex that are formed during different partial reactions, the most studied is the [2Fe-2S] cluster of the Rieske iron-sulfur protein (ISP), the EPR signal of which was found to vary with the redox state of the quinone pool, and inhibitor occupancy of the Q<sub>c</sub>-site (13–18).

Interaction of the Q<sub>c</sub>-site inhibitors stigmatellin and UHDBT with the Rieske cluster occurs via formation of a hydrogen bond with the His-161, which is one of the iron ligands of the reduced cluster (7–12). Although none of the crystallographic structures shows any occupancy of the site by the substrate ubihydroquinone (quinol, QH<sub>2</sub>) or the product ubiquinone (quinone, Q), much biophysical evidence had suggested that both these native occupants interact with the ISP through a similar configuration (2, 13–18). Previous applications in the g<sub>r</sub> region found minor effects of inhibitors on the ESEEM spectra of UHDBT-treated mitochondrial bc<sub>1</sub> complex (19), and DBMIB-treated b<sub>f</sub><sup>c</sup> complex isolated from spinach chloroplasts (20). No conclusions...

*This work was supported by National Institutes of Health Grant GM55438 (to A. R. C.); NATO Grant 977132 (to A. R. C. and R. S.); National Science Foundation Grant INT-9910113 (to S. A. D.); Ministry of Education, Science, Sport and Culture of Japan Grant-in-aid 1169237, and Japan-United States Cooperative Science Program Grant from JSPS BSAR-507 (to T. I.). The costs of publication of this article were defrayed... 

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**The abbreviations used are: cyt, cytochrome; bc<sub>1</sub>, complex, the ubi-hydroquinone-cytochrome c oxidoreductase (EC 1.10.2.2); ESEEM, electron spin echo envelope modulation; HYSCORE, hyperfine sublevel correlation; sq, single-quantum transition; dq, double-quantum transition; UHDBT, 5-n-undecyl-6-hydroxy-4,7-dioxobenzothiazole; DBMIB, 2,5-dibromo-6-methyl-3-isopropyl-1,4-benzoquinone; Q and QH<sub>2</sub>, quinone and quinol, respectively, forms of ubiquinone-10; ISP (ISP<sub>red</sub> and ISP<sub>red<sub>1</sub></sub>), Rieske iron-sulfur protein (oxidized and reduced forms).
were drawn about possible interactions of the inhibitors with the cluster or changes in geometry of ligands.

In this paper we describe initial studies using high resolution pulsed EPR to explore the involvement of the reduced ISP (ISP_red) in formation of the enzyme-product (EP) complex at the Q-site and in changes in conformation in response to the inhibitors stigmatellin and myxothiazol as alternative occupants of the site. We have used orientation-selective ESEEM experiments to quantify these effects through the hyperfine couplings of the reduced cluster with nitrogen of the coordinated histidines. We demonstrate the first quantitative characterization of the influence of the Q-site occupants on the distribution of electron spin density over the ligands and the geometry of their coordination.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Recombinant wild-type cyt bc₁ complex having the His-tag at the C terminus of cyt b subunit (BH6) was homologously overproduced in *Rhodobacter sphaeroides* cells and purified in a single-step purification procedure as described previously (21). This protocol yields an active enzyme containing four polypeptides, three to four ubiquinones, and the four redox cofactors in their stoichiometric ratios. The purified enzyme (∼20 μg per monomer) was immediately concentrated using Centricon (Amicon Inc., Beverly, MA) concentrators with a cutoff of 100,000 Da at 4 °C. Where added, all inhibitors were present in ∼9.4-fold excess concentration over the cyt bc₁ monomer. The following samples, each containing 0.01% N-dodecyl-β-maltoside (β-DM), 15 μg/ml phosphatidylcholine, and 20% glycerol (added as a cryoprotectant), and antimycin (to eliminate the Q_i-site semiquinone), were used: the form with native occupant (quinone) (0.60 mM cyt c₁1 monomer), the form with stigmatellin, or 0.31 mM), and the myxothiazol-bound form (0.42 mM).

**EPR Experiments**—EPR measurements were carried out using X-band Bruker ESP-380E spectrometer equipped with Oxford CF 935 cryostats, at 9 K. In ESEEM experiments one-dimensional three-pulse and two-dimensional four-pulse sequences were used as described in detail elsewhere (22, 23).

**RESULTS AND DISCUSSION**

**EPR Spectra**—EPR spectra of reduced samples of bc₁ complex with different occupants of the Qₐ-site show the typical anisotropic line characteristics of a rhombic g-tensor. The most noticeable difference between the spectra is in the position and width of the gₓ component (13–18, 24), which decreases from ∼1.805 in bc₁ complex with quinone at the Qₐ-site, to 1.783 with stigmatellin, or 1.785 with myxothiazol. Variations of the two other g-tensor components are less significant but readily detectable. The g-tensors correspond well with previously reported values for similar samples; in addition, we note that the values in the presence of myxothiazol are similar to those for the protelyzed ISP head domain (24). The g-tensor principal values of ISP_red, and other derived parameters, are summarized in Table IA.

Changes of g-tensor for Rieske clusters have been analyzed in terms of a ligand-field model of the orbitals of the Fe²⁺ ion in the reduced clusters (25, 26). The theory describes the variation of g-tensor in terms of a mixing of the lowest and highest d-orbitals, and a phenomenological angular function, θ, changes in which were associated with distortion of Fe²⁺ ligand field. The differences observed for the EPR spectra of the samples used in this study correspond to a variation of θ = 1°. However, this cannot be attributed to any specific structural changes of the cluster (26).

An alternative approach describes the differences between spectra using two parameters, Rₓ and Rᵧ, derived from the measured principal values of the g-tensor system through the following equation (19, 27).

\[ Rₓ = \frac{300(gₓ - gₓ\text{av})}{(2gₓ - gᵧ - gᵧ\text{av})\%} \]

(Eq. 1)

The values of Rₓ and Rᵧ depend on the complete width of the spectra characterized by gₓ – gᵧ and by gₓ – gᵧ and gᵧ – gᵧ splittings (Table IA). Rₓ and Rᵧ are equal (with a value of 100%) when gᵧ – gᵧ = gₓ – gᵧ. A surplus of the left part over the right part leads to Rₓ > 100% and Rᵧ < 100% and vice versa. Link (24) has suggested that such a transformation of the spectra, and corresponding variations of Rₓ and Rᵧ, might result from a change from z to x in the unique principal axis of the ligand field coordinate system.

Analysis of the spectra (Table IA) using this approach shows that the ISP_red in the complex with quinone or stigmatellin has values for Rₓ and Rᵧ that are opposite (relative to the 100% border) to the values in the presence of myxothiazol.

This could be interpreted as a switch in orientation of the unique principal axis in these two cases as a result of a variation in the strength of interaction of the Qₐ-site occupants with the reduced cluster, which differentially affects the ligand field and possibly the ligand geometry around the Fe(II) ion.

**ESEEM Spectroscopy**—To investigate the interaction of the Qₐ-site occupants with the ISP_red in greater detail, we have applied ESEEM spectroscopy to measure the hyperfine couplings between the cluster and the nitrogens of coordinated histidine ligands. For each sample, the three-pulse ESEEM and HYSCORE patterns were collected at three different magnetic fields, corresponding to the singularities of the g-tensor principal values in EPR spectra. Measurement of echo envelopes at different points in the anisotropic EPR spectrum selects iron-sulfur clusters with different orientations of the g-tensor relative to the applied magnetic field.

**Fig. 1** shows the superimposed plots of three-pulse ESEEM spectra (frequencies <10 MHz) of ISP_red for three bc₁ complex samples. The spectra contain a set of lines in the region 0–8 MHz attributable to local interaction with ¹⁴N nitrogens (19,
The Interaction of Rieske Cluster with $Q_o$-site Occupants

The data in Fig. 1 clearly indicate an orientation dependence of the spectra that arises from excitation of clusters differently oriented with respect to the field. As a result, at both edges ($g_x$ and $g_z$ regions) of the EPR spectra, the intensity of the ESEEM lines in the region 5–8 MHz is significantly less than the intensity of lower frequency peaks. In contrast, in the central part ($g_y$ region) the peaks at 5–8 MHz are comparable with those at low frequencies. The position of the highest frequency peak changes by about $-0.5$ MHz, which is greater than the variation caused by choice of magnetic field.

Comparison of the ESEEM spectra obtained for the three samples shows other obvious differences, especially at $g_x$ and $g_z$. Features of note in the spectra at $g_x$ include the variation of the relative intensities of two components with highest frequencies at 7.0 and 7.7 MHz, the enhancement of the line at 5 MHz in the sample with stigmatellin, and clear variations of the shapes of spectral lines at frequencies $<4.5$ MHz. Differences of a similar magnitude were observed when the field was in the $g_y$ region. These are clearly seen as variations of the maximal position and line shape of the highest component with frequency between 6.5 and 7.5 MHz and alterations of the spectral shape at $<5$ MHz. In contrast the spectra at the $g_z$ line showed only marginal variations (in the region $<5$ MHz), in confirmation of previous reports (19, 20).

The difference between the samples was also seen in the nitrogen HYSCORE spectra, which provide cross-correlations between different transitions of each nitrogen nucleus from opposite electron spin manifolds, and help in their assignment (28). They resolve also the anisotropy of nuclear transitions corresponding to $(I_{dq})$ in the $(++)$ quadrant. Fig. 2 shows a comparison of HYSCORE spectra of the reduced [2Fe-2S] cluster in the complexes with stigmatellin (A) or myxothiazol (B), measured at $g_x$. There are different intensities of all cross-peaks in the $(++)$ quadrant of the spectra. The cross-peaks spread around $(3.4, 1.5)$ MHz (indicated by 1 and 1') present in the $(++)$ quadrant of the spectrum with stigmatellin are dominant features. In contrast there are only weak traces of these peaks in the spectrum with myxothiazol.

From a combined analysis of three-pulse and HYSCORE spectra, we were able to assign pairs of $dq$ transitions for both $N_x$ nitrogens for the three samples at different field settings. Using the frequencies of the $dq$ transitions, one can estimate the diagonal elements of the nitrogen hyperfine tensor, $A_i$, in the $g$-tensor coordinate system from the second-order expressions for $v_{dq}$. Frequencies suggested in Ref. 29, $A_i = 2\nu_i/\nu_{dq_i}$ and $\nu_{dq_i} = (1 + 1/\nu_{dq_i})/\nu_{dq_i}$, values for $\nu_{dq}$ are taken from the corresponding spectra observed near $g_m$. Among two values of $A_i$ found for each orientation of magnetic field, the larger value must always be assigned to one nitrogen ($N_1$) and smaller one to another ($N_2$), as shown by electron nuclear double resonance data with $^{15}N$-labeled Rieske proteins (30).

The data of Table IB show variation of $A_i$ in samples with different $Q_o$-site occupants, either through variation in the distribution of the unpaired spin density or through a change in the geometry of ligation. Both these effects appear to contribute

**Fig. 1.** X-band three-pulse ESEEM spectra of ISP$_{o-x}$ in the presence of different $Q_o$-site occupant. top, quinone; middle, stigmatellin; bottom, myxothiazol. In each spectrum, antimycin was also present to displace the $Q_o$-site occupant. Spectra are displayed as the modulus of the Fourier transform along time $T$ axis recorded near the $g_x$ region (left), $g_y$ (center), or $g_z$ (right). The initial $\tau$ is 88 ns in the farthest trace, increased by 24 ns in the successive traces. Microwave frequency was 9.712 GHz.

**Fig. 2.** Comparison of two-dimensional ESEEM (HYSCORE) spectra of reduced [2Fe-2S] cluster in bc complex with stigmatellin (top) and myxothiazol (bottom). Excitation was at the $g_x$ line. Myxothiazol (microwave frequency 9.717 GHz, $\tau=128$ ns). Stigmatellin (microwave frequency 9.711 GHz, $\tau=128$ ns).
to the differences seen on interaction with the different occupants.

Direct information about the unpaired 2s orbital spin density on the nitrogen follows from the isotropic hyperfine constant (22), which is equal \( A_{\text{iso}} = A_x + A_y + A_z / 3 \) (Table IB). The point of particular interest is the increase \(-10\% \) of the \( A_{\text{iso}} \) on N1, but not on N2. The change of \(-0.5 \text{ MHz} \) is significantly greater than the range of scatter for the experiment.

The three-pulse ESEEM spectrum of bc1 with myxothiazol at \( g_z \) shows a triplet with frequencies 6.8, 3.4, and 1 MHz produced by two N\(_2\)-atoms magnetically equivalent along this direction. This spectrum provides two sets of nuclear frequencies, at 6.8, 3.4, and 3.4 MHz and at 3.4, 1.7, and 1 MHz (confirmed by HYSSCORE data), which are an indication of zero quadrupole splitting along \( g_x \) for both nitrogens. No equivalence between these nitrogens is found for the samples with stigmatellin or quinone at either \( g_x \) or \( g_z \), so this difference in symmetry between the two N\(_2\)-atoms cannot be attributed to a switch of unique axis. The multiple lines indicate the presence of quadrupole splittings from at least one nitrogen along these axes. Because the orientation of the N\(_5\) quadrupole tensor is associated with the orientation of the imidazole ring, this demonstrates a change in orientation of a histidine in these samples compared with that in the complex with myxothiazol.

**Biochemical Relevance**—In the structure of the bc1 complex with stigmatellin, the hydrogen bond (\(-2.8 \text{ Å} \) in the yeast complex, 1exv) involves the carbonyl oxygen of stigmatellin, and the N\(_5\) of His-161, which provides a ligand to the cluster through N\(_5\). This interaction (Fig. 3) confirmed a large body of previous physicochemical evidence, suggesting a liganding between ISP\(_{\text{red}}\) and UHDBT or stigmatellin (reviewed in Refs. 2).

The 1998 Nature (Lond.) 392, 677–684

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