Spectroscopic identification of the axial ligands of cytochrome b_{560} in bovine heart succinate-ubiquinone reductase

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Abstract The axial ligands of low potential cytochrome b_{560} in the five subunit bovine heart succinate-ubiquinone reductase complex and in the isolated quinone binding proteins have been investigated using EPR and near-infrared magnetic circular dichroism spectroscopies. The results are consistent with bis-histidine ligation with near-perpendicular imidazole rings for cytochrome b_{560} in the four-subunit complex. The pronounced changes in EPR properties that accompany isolation of the cytochrome-b_{560} containing quinone binding proteins, are attributed to perturbation of the orientation of the imidazole rings of the heme bis-histidine ligands, rather than a change in axial ligation.

Key words: Succinate-ubiquinone reductase; Succinate dehydrogenase; Quinone binding protein; Cytochrome b_{560}; Electron paramagnetic resonance; Magnetic circular dichroism

1. Introduction

Bovine heart succinate-quinone reductase (SQR) is a membrane-bound component of the mitochondrial respiratory chain. It catalyzes the oxidation of succinate to fumarate and the reduction of ubiquinone, thereby contributing electrons ultimately to oxidative phosphorylation and energy transduction. The purified complex can be resolved into two reconstitutively active fractions [1,2]. One fraction, composed of the two larger subunits (70 and 27 kDa), is the soluble succinate dehydrogenase (SDH) which contains a flavin moiety as well as three Fe-S clusters. The other fraction, termed the quinone binding proteins (QPs), anchors SDH to the mitochondrial inner membrane and comprises three smaller subunits (14, 11, and 9 kDa) [3,4]. This membrane-bound fraction contains the cytochrome b_{560} that is the focus of this paper [5,6].

As their name suggests, the QPs facilitate interaction of SDHs and the highly homologous fumarate reductases (FRDs) with quinones. However, a specific role for the heme group in mediating electron transfer to or from quinone has yet to be established. Indeed QPs from SQRs and quinol-fumarate reductases (QFRs) show little sequence homology, as well as variability in the number of subunits and the number and midpoint potential of the heme groups [1,2]. For example the QPs in QFR from *E. coli* comprise two subunits (15 kDa and 13 kDa), but are devoid of a *b*-type cytochrome [7]. The QPs in SQRs from bovine heart mitochondria [8,9] and *E. coli* [10,11] both contain one cytochrome *b*, but differ in the number and size of the constituent subunits (14-, 11- and 9-kDa subunits for bovine heart QPs [3,4]; 14- and 13-kDa subunits for *E. coli* QPs [12,13]). Moreover, the cytochrome *b*s in these enzymes differ greatly in their midpoint potentials; E_m = +36 mV and −180 mV for *E. coli* and bovine heart SQR, respectively. In the SQR from *Bacillus subtilis* [14] and QFR from *Wolinella succinogenes*, [15], the membrane anchor consists of a single subunit (22–30 kDa) which contains both a low and a high potential cytochrome *b*.

EPR and magnetic circular dichroism (MCD) spectroscopies have been used to investigate the axial ligation of cytochrome b_{558} from *B. subtilis* and *E. coli* SQR [16,17]. In both cases the data was interpreted in terms of *bis*-histidyl axial ligation with near-perpendicular orientation of the imidazole rings responsible for the highly anisotropic low spin (HALS) EPR signals. However, this was before the presence of distinct low and high potential hemes was established in *B. subtilis* cytochrome b_{558} [14]. Therefore, although these results taken together attest to bis-histidyl ligation for the high potential cytochrome *b*s (characterized by HALS EPR signals with ramped line shapes for the low-field component), the axial ligation of the low potential cytochrome *b*s remains ill-defined. The low potential cytochrome b_{560} of bovine heart SQR exhibits a HALS-type EPR signal with a Gaussian-shaped low-field component at g = 3.46, but this resonance is replaced by two new resonances with low field g-values at 3.07 and 2.92 in purified QPs [8]. In this work, the two complementary techniques of EPR and MCD have been used to investigate the axial ligation of the low potential cytochrome b_{560} in bovine heart SQR and the isolated QPs.

2. Materials and methods

Bovine heart SQR and QPs were purified and assayed as previously described [8]. Samples for spectroscopic measurements were prepared in 50-mM Tris-succinate D_2O buffer, pH 7.4 with 50% (v/v) glycerol and containing 0.5% sodium cholate. The sample concentrations used in quantifying the MCD spectra are based on the cytochrome concentration as assessed by the differential reduced minus oxidized extinction coefficient at 560 nm minus 575 nm, $\Delta\varepsilon$ = 28.5 $mM^{-1} \cdot cm^{-1}$ [8]. Identical samples were used for EPR and MCD investigations.

Absorption spectra were recorded in 1-mm cuvettes using a Hewlett Packard 8452A diode array spectrophotometer. Near-infrared and UV/vis MCD spectra were recorded using Jasco J-730 and J-500 spectrophotometers, respectively, interfaced to an Oxford Instruments SM3 superconducting magnet (magnetic field, 0–5 T; sample temperature 1.5–300 K) using the protocols described elsewhere [18]. EPR spectra were recorded on a Bruker Instruments ESP 300D X-band spectrometer equipped with an Oxford Instruments ESR 900 liquid helium flow cryostat.

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Abbreviations: SQR, succinate-quinone reductase; QFR, quinol-fumarate reductase; QP, quinone-binding protein; SDH, succinate dehydrogenase; FRD, fumarate reductase; HALS, highly anisotropic low spin; MCD, magnetic circular dichroism.

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3. Results

The combination of EPR and MCD spectroscopies provides a powerful and convenient method for assessing axial ligation of low spin Fe(III) hemes [19–22]. Low temperature greatly enhances the sensitivity of MCD spectroscopy for detecting low spin Fe(III) hemes and facilitates investigation under analogous conditions to those required for EPR measurements. However, low temperature MCD requires the presence of a glassing agent such as 50% (v/v) glycerol and studies of the effect of this medium on the EPR properties are a prerequisite for meaningful MCD investigations. Fig. 1 shows the EPR spectra for the samples of bovine heart SQR and QPs used for the low temperature MCD studies. The spectra are identical to those published previously in the absence of glycerol [8]. The cytochrome *b* in SQR is characterized by a HALS-type low-spin Fe(III) resonance with a Gaussian-shaped low-field component centered at $g = 3.46$ signal. On isolation of the QPs, this is replaced by two new low-spin Fe(III) resonances of comparable intensities with Gaussian-shaped low-field components centered at $g = 3.07$ and 2.92. Residual amounts of these resonances are also apparent in the SQR samples. The relative intensities of the $g = 3.46$, 3.07, and 2.92 signals were not significantly altered in spectra recorded at 9 K with 1-mW microwave power indicating that none are saturated under the conditions used in figure 1.The isotropic signals at $g = 4.3$ are attributed to adventitiously bound high spin Fe(III) ion and both samples exhibited weak signals at $g = 6.0$ arising from negligible amount of high spin Fe(III) heme (not shown).

The dramatic changes in the EPR properties of cytochrome b_{560} that accompany isolation of QPs result in only minor changes in the low temperature MCD spectrum in the UV/visible region, see Fig. 2. The spectra of both SQR and QPs in the 300–600 nm region are quite characteristic of low spin Fe(III) hemes and MCD magnetization studies for the dominant bands confirm a $S = 1/2$ ground state (data not shown). Since these MCD bands are porphyrin $\pi \rightarrow \pi^*$ transitions, it therefore unlikely that changes in the porphyrin macrocycle or its substituents are responsible for differences in EPR properties. SQR does have an additional positive MCD band centered at around 720 nm, but comparison with succinate-treated samples of reconstitutively preparations of soluble bovine SDH [23] and magnetization studies (data not shown) identify this band as originating primarily from the $S = 2$ $[3Fe\text{-}4S]^0$ cluster (reduced center 3) of SDH. Likewise contributions from the paramagnetic reduced $[3Fe\text{-}4S]^0$ and $[2Fe\text{-}2S]^+$ of SDH that are present in succinate-reduced SQR are responsible in part for the differences in the MCD in the 500–600 nm region. The absence of any indication of a '695-nm' band in the low temperature MCD or absorption spectrum of the QPs argues against an axially coordinated methionine residue.

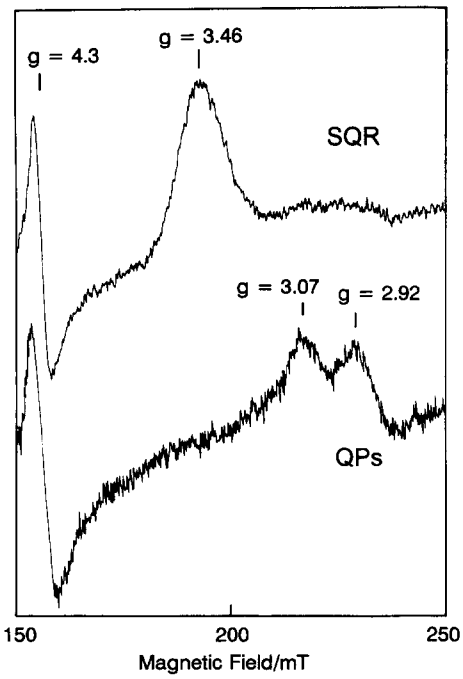

Fig. 1. X-band EPR spectra of bovine heart SQR and QPs. The samples (0.14 mM for SQR and 0.04 mM for QPs) were in 50-mM Tris-succinate D_2O buffer, pH 7.4 with 50% (v/v) glycerol and 0.5% sodium cholate. EPR conditions: temperature, 9 K; microwave power, 20 mW; modulation amplitude, 1.0 mT; microwave frequency, 9.45 GHz.

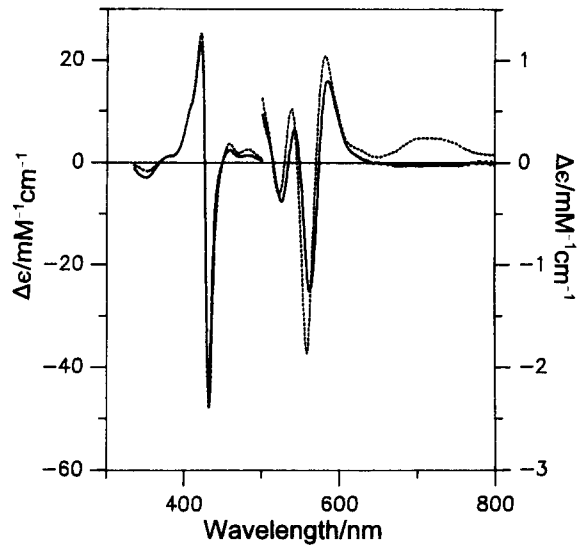

Fig. 2. UV/Visible MCD spectra of bovine heart SQR (broken line) and QPs (solid line). The samples conditions are as described in Fig. 1. The MCD spectra were recorded at 4.2 K with a magnetic field of 5 T.

While the UV/visible MCD is relatively insensitive to the nature of the heme axial ligands, the energy and intensity of the porphyrin (π)-to-iron charge MCD bands in the near-IR region have been found to be extremely sensitive [19–22]. This is to be expected since the nature and relative orientation of the axial ligands will have a direct affect on the energies of the Fe d-orbitals. The low-temperature near-IR MCD spectra for SQR and QPs, see Fig. 3, conform to the generally observed features of low spin Fe(III) hemes, i.e. two positive MCD bands with the lower energy component being more intense and the higher energy component exhibiting partially resolved structure. The most intense band occurs at 1600 nm ($\Delta\varepsilon = 390\ M^{-1} \cdot cm^{-1}$ at 4.2 K and 5 T) in SQR and is broadened and shifted to 1535 nm ($\Delta\varepsilon = 270\ M^{-1} \cdot cm^{-1}$ at 4.2 K and 5 T) in the QPs.

On the basis of studies of a wide range of structurally defined low spin Fe(III) hemoproteins and synthetic analogs, wavelength ranges for the most intense near-IR MCD band (λ_{CT}) have been established for each of the known types of heme axial

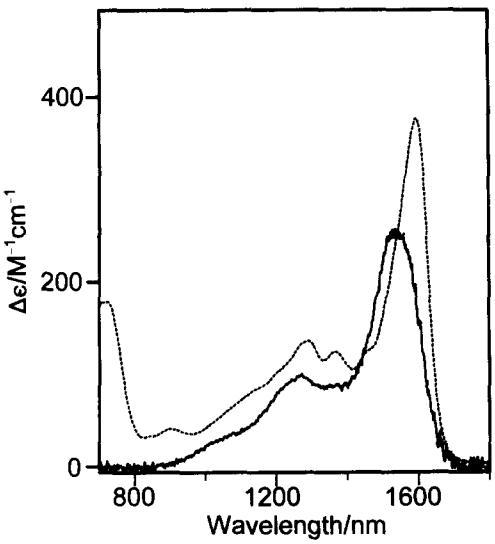

Fig. 3. Near-IR MCD of bovine heart SQR (broken line) and QPs (solid line). The samples conditions are as described in Fig. 1. The MCD spectra were recorded at 4.2 K with a magnetic field of 5 T.

ligation [19–22]. This criterion alone definitively rules out the possibility of His/Met or Met-Met axial ligation (λ_{CT} = 1720–1930 nm and 2120–2280 nm, respectively), but leaves open the possibility of His/His, His^-/Met, His/amine (Lys or N-terminal amine) ligation for cytochrome b_{560} in both SQR and QPs. The intensity of the near-IR MCD and the lack of a '695-nm' band argue against His^-/Met coordination. His/His (or His/imidazole) ligation results in λ_{CT} = 1500–1640 nm and the intensity is variable, depending on the relative orientation of the imidazole rings. Values of $\Delta\varepsilon$ as high as 400 $M^{-1}\cdot cm^{-1}$ at 4.2 K and 5 T have been reported and are attributed to near-perpendicular orientation of the imidazole rings [16,20]. Well established examples His/amine axial ligation are much less abundant, but are provided by alkaline horse heart cytochrome *c* (His/Lys) [24], cytochrome *f* (His/N-terminal amine) [25], and the butylamine adduct of leghemoglobin [19]. Alkaline cytochrome *c* has λ_{CT} = 1480 nm with $\Delta\varepsilon$ = 420 $M^{-1}\cdot cm^{-1}$ (4.2 K and 5 T) [26], cytochrome *f* has λ_{CT} = 1520 nm with $\Delta\varepsilon$ = 675 $M^{-1}\cdot cm^{-1}$ (4.2 K and 5 T) [27], and butylamine leghemoglobin has λ_{CT} = 1550 nm with $\Delta\varepsilon$ = 330 $M^{-1}\cdot cm^{-1}$ (4.2 K and 5 T) [19]. While there is currently no precedent, it is conceivable that His/amine axial ligation could result in λ_{CT} values as high as 1600 nm. However, based on the currently available data, the near-IR MCD properties of cytochrome b_{560} in SQR are clearly most consistent with His/His axial ligation. On the basis MCD data alone, it is not possible to decide between His/His and His/amine ligation for cytochrome b_{560} in QPs.

4. Discussion

The primary objectives of this work was twofold: to establish the axial ligation of cytochrome b_{560} in SQR and to investigate the origin of the changes in properties of this prosthetic group that accompany isolation of the QPs. The combination of low temperature EPR and MCD results limits the possibilities for the axial ligation of cytochrome b_{560} in SQR to His/His or His/amine (Lys or N-terminal amine), and strongly favors the former assignment. The reasoning behind this conclusion is as follows. In the absence of exogenous ligands such as CN^-, low spin Fe(III) hemes with HALS-type EPR signals (broadly defined as resonances with $g > 3.1$ low-field components) have only been reported for His/His, His/amine (Lys or N-terminal amine), and His/Met axial ligands. His/Met axial ligation for the cytochrome b_{560} in SQR which has a Gaussian-shaped low field component at g = 3.46, is ruled out by the absence of a '695-nm' band in the absorption or MCD spectra and by the wavelength of the porphyrin (π)-to-Fe charge transfer transition in the near-IR MCD spectrum, λ_{CT} = 1600 nm. As noted above, the observed ranges of λ_{CT} overlap for His/His and His/amine axial ligation (1500–1640 nm and 1480–1550 nm, respectively [19]), and discrimination between these possibilities has largely centered on the shape and location of the low-field component of the EPR resonance. The three known examples of His/amine axial ligation all exhibit EPR signals with Gaussian-shaped low-field components at $g \geq 3.3$. His/His ligation usually results in a low-field component at $g < 3.3$, except in cases where near perpendicular imidazole rings creates near-axial symmetry. In these instances the line-shape often becomes non-Gaussian, with a sharp cut off on the low-field edge, resulting a ramped line shape [28]. Hence a ramped line shape for the low-field EPR feature with $g > 3.3$, coupled with λ_{CT} in the range 1500–1640 nm is considered indicative of His/His axial ligation. However, since there are well-documented examples of bis-histidine *b*-type cytochrome with HALS-type EPR signals and Gaussian-line shapes for the low field resonance (e.g. the g = 3.44 low potential cytochrome *b* of bovine heart ubiquinol-cytochrome *c* oxidoreductase [29]), His/amine axial ligation for cytochrome b_{560} in bovine-heart SQR cannot be inferred from the Gaussian-line shape. Rather, the spectroscopic data strongly favor His/His ligation based on the observation that λ_{CT} is outside the range observed for the limited examples of His/amine ligation investigated thus far. Near-perpendicular imidazole rings would then account for the high near-IR MCD intensity and the HALS-type EPR signal [20].

The spectroscopic results presented here for the cytochrome b_{560} of bovine SQR complement those obtained previously for the cytochrome *b* components of *B. subtilis* and *E. coli* SQR [16,17]. On the basis of the wavelength of the dominant charge transfer band in the near-IR MCD spectra, λ_{CT} = 1600 nm, and the ramped-shaped HALS-type EPR resonance with a low field maximum at g = 3.68, it can be concluded that the high potential cytochrome *b*s in both enzymes have bis-histidyl ligation. Although the existence of distinct low and high potential cytochrome *b*s in *B. subtilis* cytochrome b_{558} was not clearly apparent at the time of the EPR and MCD studies [16], it is now evident that the low potential cytochrome *b* in this enzyme has properties almost identical to those of cytochrome b_{560} of bovine heart SQR, i.e. HALS-type EPR signal with Gaussian-shaped low-field component centered at g = 3.42 and $\lambda_{CT} \approx$ 1600 nm [14,16]. Moreover, mutagenesis studies of specific histidine residues in *B. subtilis* cytochrome b_{558} have identified four histidines in hydrophobic segments that are essential for heme ligation and correct assembly of SQR [30]. Hence the available mutagenesis data provides additional support for a His/His axial ligand assignment for the low potential cytochrome *b* in *B. subtilis* SQR and this support can be extended to the cytochrome b_{560} of bovine heart SQR in light of the analogous physical properties of these heme groups. Thus far amino acid sequence data based on the nucleotide sequence is only availa-

ble for the larger of the three QP subunits of bovine heart SDH [9]. While there are two histidine residues that are conserved among the equivalent QPs of bovine heart, *E. coli* and *B. subtilis*, at present it is not clear if the cytochrome *b* bridges across membrane spanning helices within this subunit or between the two QP subunits.

Isolation and detergent solubilization of bovine heart QPs results in loss of the '$g = 3.46$' resonance and the appearance of two new resonances in approximately equal concentrations with low field *g*-values at 3.07 and 2.92. Previous EPR studies revealed that only the '$g = 3.07$' species reverts back to the '$g = 3.46$' species on reconstitution with SDH to form SQR [8]. It is not possible to distinguish these two low spin Fe(III) heme species in the near-IR MCD spectra and both exhibit λ_{CT} values close to 1535 nm which is characteristic of either His/His or His/amine axial ligation. However, since all know examples of His/amine axial ligation have low-field *g*-values ≥ 3.3, the EPR argues in favor of bis-histidine axial ligation for both species. The decrease in near-IR MCD intensity and the smaller *g*-value anisotropy compared to intact SQR are therefore attributed to increased rhombicity associated with relaxation of the near-perpendicular arrangement of coordinated imidazoles [20]. This is consistent with movement of the trans-membrane α-helices of the QPs on dissociation of the SDH subunits. EPR signals with a low field component at $g = 2.9$ are commonly observed for irreversibly denatured membrane-bound *b*-type cytochrome [31], and very similar EPR signals are observed for bis-imidazole model complexes [28], i.e. when the constraints of protein conformation are completely relaxed.

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