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Identification of the axial heme ligands of cytochrome b_{556} in succinate: ubiquinone oxidoreductase from *Escherichia coli*

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Abstract Electron paramagnetic resonance (EPR) and near-infrared magnetic circular dichroism (MCD) have been used to identify the ligands to the cytochrome b_{556} component of succinate: ubiquinone oxidoreductase (succinate dehydrogenase) from *Escherichia coli*. The 'highly axial low spin' (HALS) EPR spectrum suggests bis(histidine) ligation of the heme with the histidines in a staggered configuration. The near-infrared MCD spectrum exhibits a low energy maximum at 1600 nm which is also clearly indicative of bis(histidine) ligation of the heme iron. The data unambiguously demonstrate that the heme b_{556} is ligated to *E. coli* succinate dehydrogenase *via* two histidines.

Key words: Heme protein; Axial ligand; EPR; MCD; Succinate dehydrogenase

1. Introduction

Succinate:ubiquinone oxidoreductase (complex II, succinate dehydrogenase) from *Escherichia coli* contains four non-identical subunits encoded by the *sdhCDAB* operon [1,2]. SdhA and SdhB are hydrophilic subunits which contain the succinate binding site (SdhA), a covalently attached FAD (SdhA), and three [Fe-S] clusters (SdhB). SdhC and SdhD are small hydrophobic subunits that anchor the hydrophilic SdhAB subunits to the inner surface of the cytoplasmic membrane (see [3]). The purified *E. coli* enzyme contains, in addition, a single cytochrome component, cytochrome b_{556}, which is associated with the anchor polypeptides [4–6]. It is generally believed [3] that the SdhCD hydrophobic polypeptides provide the ubiquinone binding site and that cytochrome b_{556} is likely to facilitate electron transfer to ubiquinone in the cytoplasmic membrane *via* one or more of the [Fe-S] clusters in SdhB. It is the goal of the current work to identify the nature of the axial ligands of cytochrome b_{556}, to provide the basis for site-directed mutagenesis studies to identify the specific amino acid residues that are the axial ligands within SdhC and/or SdhD.

Succinate dehydrogenase complexes from several sources have been characterized and are always associated with cytochrome *b* components [3]. The enzyme from bovine [7], *Paracoccus denitrificans* [8] and *E. coli* [4] are associated with a single heme B prosthetic group, whereas that from *Bacillus subtilis* contains two cytochrome *b* components [9]. All of the succinate dehydrogenases contain homologues of the hydrophilic SdhA and SdhB subunits, but in some instances these are anchored to the membrane through an interaction with a single, large hydrophobic subunit instead of the two small hydrophobic subunits (e.g., SdhCD). This is the case of the succinate dehydrogenase complex from *B. subtilis*.

The only published studies specifically directed at identifying the heme ligands within this family of enzymes have been those with the succinate dehydrogenase from *B. subtilis* [10,11]. A combination of EPR and near-infrared MCD spectroscopies [12] have shown that each of the hemes associated with the hydrophobic subunit (cytochrome b_{558}) of this enzyme has bis(histidine) ligation [11]. Subsequent site-directed mutagenesis studies have resulted in a postulated model in which specific histidines within this subunit have been assigned to the cytochrome components [10]. The data in this paper show that the single protoheme component of the *E. coli* succinate dehydrogenase also has bis(histidine) ligation.

2. Materials and methods

E. coli succinate dehydrogenase was prepared as previously described using the detergent Lubrol-PX [4]. The enyzme was concentrated in deuterium oxide (D_2O) for the spectroscopic measurements in the following manner. An 18-ml sample of the purified enzyme (5.65 mg/ml) was concentrated to approximately 2.5 ml using an Amicon PM-30 filter. The sample volume was brought to 15 ml using buffer prepared with D_2O containing 0.2% Lubrol-PX, 1 mM EDTA, 40 mM Tris-Cl, pD 7.55 (room temperature). This concentration and buffer exchange was repeated four times. The sample was then centrifuged at $460{,}000 \times g$ for 17 h at 4°C. This procedure resulted in a dark red-brown pellet containing the enzyme. After resolubilization, the enzyme was active, as measured by both succinate:ferricyanide and succinate:ubiquinone oxidoreductase assays [4]. Reported measurements of pD are simply pH meter reading +0.4 in accordance with the suggestion of Glasoe and Long [13]. In order to obtain optical quality glasses upon freezing, aqueous samples were diluted in d_6 ethanediol (55%, v/v). Deuterated reagents were obtained from Cambridge Isotope Laboratories. The concentration of protoheme in samples was determined by the pyridine hemochromogen method [14].

EPR spectra were obtained using a hybrid instrument consisting of a Varian E109E console, used to provide the field modulation to a Bruker B-E 25 magnet, with an EPR 082 power supply and B-H 15 field controller, plus a Varian E1-2 microwave bridge and V-453–3 cylindrical cavity. The spectrometer was fitted with an Oxford Instruments ESR 900 liquid helium flow cryostat and 3120 temperature controller.

MCD spectra were recorded using an Aviv Associates 41DS circular dichroism spectrometer and Cryomagnetics Incorporated cryomagnet. A 'single spectral scan' consists of data recorded with the applied field in the forward direction minus the reverse field data, the difference being divided by two. In this manner, contributions arising from natural circular dichroism are subtracted from the spectrum.

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Abbreviations: EDTA, ethylenediaminetetracetic acid; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); MCD, magnetic circular dichroism.

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3. Results

The X-band EPR spectrum of pelleted material before redissolution in buffer is shown in Fig. 1. The dominant feature at g = 2.01 is typical of oxidized Fe_3S_4 clusters and has been observed previously [4]. The very weak signal at g = 3.63 is in the reported range for the g_z component of mitochondrial *b*-type cytochromes, whereas in the present case, g_x and g_y are not observable [15,16]. Thomson and co-workers [12,17,18] have shown that this 'highly axial low spin' (HALS) spectrum corresponds to a situation where two axial histidine ligands to the ferric heme are in a staggered conformation, i.e. their imidazole rings are oriented at about 90° with respect to each other.

The near-infrared MCD spectrum of the dissolved pellet exhibits a low energy maximum at 1600 nm (Fig. 2) clearly indicative of bis(histidine) coordination to the heme [12,19] and quite in keeping with the EPR data. Taken together, these two pieces of spectral data are unambiguous concerning this assignment.

4. Discussion

The data definitively show that the heme b_{556} component of *E. coli* succinate dehydrogenase is ligated to the protein by two histidine residues. This is, perhaps, not surprising considering the previous result obtained with *B. subtilis* succinate dehydrogenase [11]. However, the extrapolation from the *B. subtilis* enzyme to that of *E. coli* is not trivial. The *B. subtilis* enzyme contains two cytochrome *b* components bound to a single membrane-spanning subunit [11], whereas the *E. coli* enzyme contains a single cytochrome and two hydrophobic subunits [4]. Furthermore, there is virtually no sequence similarity apparent in comparisons of the hydrophobic subunits of the two species [3].

Bovine complex II (succinate dehydrogenase) also contains two hydrophobic subunits and a single heme, similar to the *E. coli* enzyme [3]. Recent studies have shown that the cytochrome *b* component of the bovine enzyme is also bis(histidine) (M. Johnson, C.-A. Yu, L. Yu, and B. Crouse, unpublished).

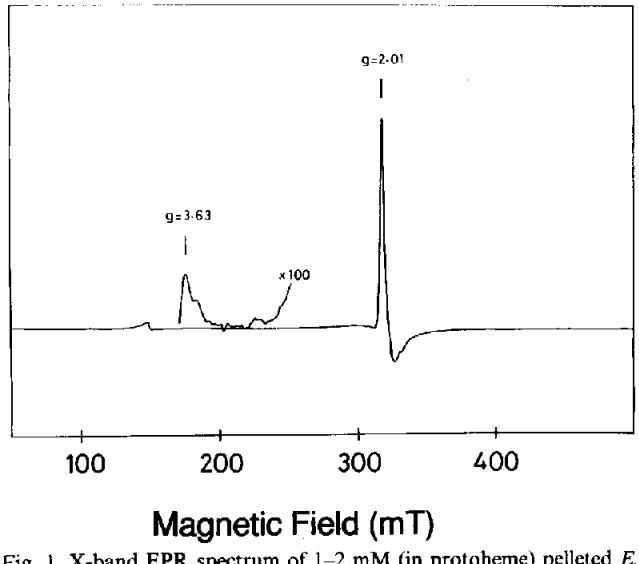

Fig. 1. X-band EPR spectrum of 1–2 mM (in protoheme) pelleted *E. coli* succinate dehydrogenase. Recording conditions: 11 K, 8.91 GH_z, 2 mW microwave power, 20 G modulation amplitude, 5×10^2 amplifier gain.

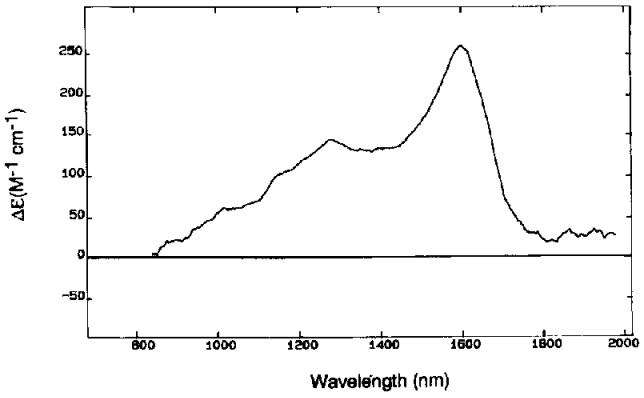

Fig. 2. MCD spectrum 0.13 mM (protoheme) *E. coli* succinate dehydrogenase, pD 7.8, in 18 mM HEPES, 0.1% (w/w) Lubrol-PX, 55% (v/v) d_6 ethanediol. Recording conditions: 4.2 K, 7.0 T, 0.9 mm pathlength, 6–12 nm spectral bandwidth, mean of 2 spectral scans.

Hence, it is likely that bis(histidine) ligation is common to all the cytochrome components in succinate dehydrogenases, despite the lack of apparent sequence similarities in the heme-containing membrane-anchor subunits.

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