

Potentiometric titration of cytochrome-*bo* type quinol oxidase of *Escherichia coli*: evidence for heme-heme and copper-heme interaction

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The cytochrome-*bo* quinol oxidase of *Escherichia coli* contains a high-spin *b*-type heme (cytochrome *o*), a low-spin *b*-type heme (cytochrome *b*) and copper. The EPR signal from cytochrome *o* is axial high spin and when titrated potentiometrically gives a bell-shaped curve. The low-potential side of this curve (E_m , approx. 160 mV) corresponds to the reduction/oxidation of the cytochrome. The high-potential side (E_m , approx. 350 mV) is proposed to be due to reduction/oxidation of a copper center; in the CuII form tight cytochrome *o*-copper spin coupling results in a net even spin system and loss of the EPR spectrum. Optical spectra of the α -bands of the reduced cytochromes at 77 K show that cytochrome *b* has its maxima at 564 nm when cytochrome *o* is oxidized but that this shifts to 561 nm when cytochrome *o* (max. 555 nm) is reduced. Both a heme-copper (cytochrome *o*-CuII) and a heme-heme (cytochrome *o*-cytochrome *b*) interaction are indicated in this quinol oxidase. These results indicate that cytochrome-*bo* quinol oxidase has a binuclear heme-copper catalytic site and suggest striking structural similarity to subunit I of the cytochrome *aa₃* system.

Quinol oxidase; Potentiometric titration; Heme-heme interaction; Coppers-heme interaction

1. INTRODUCTION

Wild-type *E. coli* are capable of synthesizing two different terminal quinol oxidases. These two oxidases, cytochrome *bo* and cytochrome *bd*, are differentially induced, apparently in response to the O₂ tension (for reviews see [1–3]). Cytochrome *o* (here the 'o' stands for oxidase) was discovered simultaneously in *E. coli* and in other bacteria by photo-chemical action spectra of CO inhibited respiration [4,5]. The CO and O₂ binding pigment of the cytochrome *bo* is the *o*-moiety; this is also a *b*-type cytochrome.

The cytochrome *bo* complex contains four subunits. DNA sequence analysis has revealed

homology both with bacterial and mammalian cytochrome *aa₃*. The *E. coli* cytochrome *o* subunit I has substantial homology with the mammalian cytochrome *aa₃* subunit I (Gennis, R.B. and co-workers, personal communication). This is the subunit of cytochrome-*c* oxidase implicated in binding the hemes and one of the coppers [6]. The subunit IV from cytochrome *o* is similar to subunit III of the *aa₃*-type oxidase. Both cytochrome-*c* oxidase and ubiquinol oxidase cytochrome *bo* contain copper and high- and low-spin hemes [7,8]. In the former, the two hemes are of the *b*-type; in the latter, they are *a*-type.

In this paper we report potentiometric analyses of the cytochrome *bo* in a strain of *E. coli* which lacks the cytochrome *bd*, but does not have amplified expression levels of the cytochrome *bo*. Evidence for both a heme-heme interaction and a heme-copper interaction, akin to those found in the mammalian cytochrome *aa₃*, is presented.

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2. MATERIALS AND METHODS

2.1. Growth of cells and preparation of membrane particles

E. coli G0103, which lacks the quinol oxidase cytochrome *bd* was obtained from the laboratory of Professor R.B. Gennis, University of Illinois, Urbana, and grown with aeration at 37°C in 201 bottles. The medium consisted of 0.5% glycerol, 0.1% cas-amino acids, 50 mM K_2HPO_4 , 16 mM $(NH_4)_2SO_4$, 2 mg·l⁻¹ thiamine, 50 μM Mo/Se supplement and 1 ml·l⁻¹ of a trace metals solution [9] adjusted to pH 7.2.

Cells were harvested in late exponential phase and cytoplasmic membrane particles were isolated by differential centrifugation following French Pressure cell treatment and stored as previously described [10].

2.2. Potentiometric redox titrations

Redox titrations were performed as described by Dutton [11] in 50 mM Tes, 2 mM EDTA buffer, pH 7.0. The oxidation reduction mediators and buffers used at 100 μM were: tetrachlorohydroquinone, *N,N,N',N'*-tetramethylenephénylene-diamine, dibromophenol-indophenol, phenazine methosulphate, 1,2-naphthoquinone and ascorbate. Ferricyanide was employed as oxidant; dithionite was used as reductant.

2.3. EPR spectroscopy

EPR spectra were obtained using a Bruker ER200D electron paramagnetic resonance spectrometer (Bruker Analytische Messtechnik GmbH, Silberstreifen, D-7512 Rheinstetten 4, FRG) equipped with a variable temperature cryostat and liquid helium transfer line (Oxford Instruments, Osney Mead, Oxford, England).

2.4. Assays

Optical difference spectra were obtained at 77 K by a split-beam instrument constructed in the Biochemistry and Microbiology department of the University of St. Andrews. Difference spectra could be obtained between samples in EPR tubes.

Protein determinations were carried out by the Lowry method modified by the inclusion of 1% (w/v) SDS to solubilize membrane bound proteins.

3. RESULTS

3.1. Electron paramagnetic resonance spectra of the cytochromes

EPR spectra of cytoplasmic membrane particles obtained at 9 K are shown in fig.1; the sample are poised at different redox potentials. A high-spin component is visible around $g = 6.0$, while a low-spin cytochrome contributes features at $g = 3.0$ (3.03) and $g = 2.25$. The $g = 4.3$ region of the spectra (not shown) is obscured by a signal from low symmetry non-heme FeIII, while the $g = 2.0$ region is overlapped by signals from CuII, iron-sulfur centers and free radicals.

The amplitude of the high-spin ferric heme signal at $g = 6.0$ increases as the redox potential is

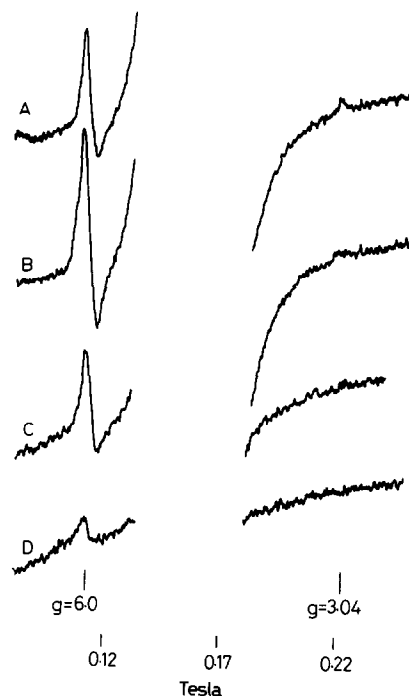


Fig.1. Electron paramagnetic resonance spectra. Samples from a potentiometric redox titration at pH 7.0 were poised at 380 mV (A), 260 mV (B), 150 mV (C) and 50 mV (D) as described in section 2. The protein concentration was 26 mg·ml⁻¹. EPR conditions: temperature 9 K; microwave frequency, 9.47 GHz; microwave power, 2 mW; modulation amplitude, 0.002 T; modulation frequency, 100 kHz.

lowered from 380 mV (fig.1A) to 260 mV (fig.1B); thereafter the signal amplitude declines with decreasing redox potential. The $g = 3.0$ and $g = 2.25$ signals (not shown), corresponding to the low-spin ferric heme, decrease monotonically as the potential is lowered. The spectroscopic conditions for the spectra shown in fig.1 were chosen as a compromise giving reasonable detection of both the high- and low-spin features in a single scan. The plots of the data shown in fig.2 were obtained from spectra where the two species were separately optimized.

3.2. Potentiometric analysis of the components

Shown in fig.2 are plots of the titration of the magnitude of the EPR signals against redox potential (E_h). In fig.2A, the behavior of the high-spin $g = 6.0$ center is shown. At the lower end of the E_h scale, a minor component (approx. 10%) does not

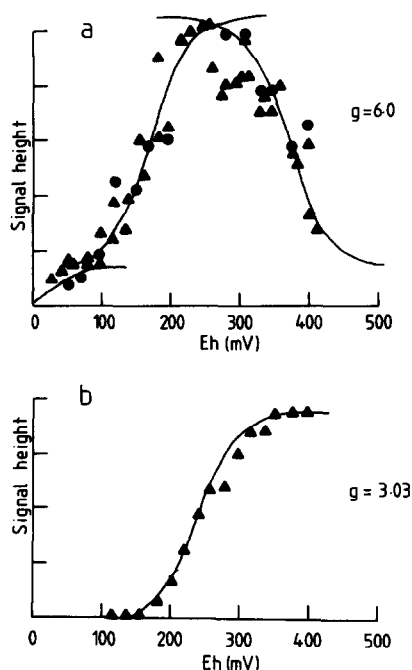


Fig. 2. Plot of redox titration data. The signal heights of the $g = 6.0$ signal (a) and the $g = 3.03$ signal (b) are plotted against E_h . The redox titrations were performed as described in section 2. The results shown in the top graph are a compilation of four titrations. The triangles indicate points on a reductive titration and the circles points on an oxidative titration. The protein concentrations were from 13 to 26 $\text{mg} \cdot \text{ml}^{-1}$. Each curve represents the contribution of a theoretical $n = 1$ component.

titrate above 40–80 mV; this may be associated with components other than cytochrome *o*. On increasing the potential, a large increase in signal height is observed corresponding to the oxidation of a high-spin ferriheme with a half point at approx. 160 mV. On raising the potential further, the signal declines, the midpoint potential of this latter wave being approx. 350 mV. The curves drawn on fig. 2A represent single $n = 1$ phases but the scatter of the data and the presumed overlap and antagonism of the two major features of the titration make more detailed analysis of the titration difficult. Redox cooperativity cannot be ruled out on the basis of these data.

Fig. 2B illustrates the corresponding titration of the $g = 3.03$ signal. Again, although the titration can be described reasonably well by a single $n = 1$ component with a midpoint potential of 250 mV, the quality of the data does not exclude multi-

phasicity. No decrease of signal amplitude at high potential was observed for this component. When redox titrations were conducted under a CO atmosphere (not shown), the high-spin feature was abolished and the low-spin component appeared unaltered.

3.3. Changes in the optical spectra corresponding to the EPR spectra

Optical spectra at 77 K were obtained using the same samples in the EPR tubes. Scans of difference spectra from 520 to 580 nm showing the heme α -bands are presented in fig. 3. Fig. 3A shows the 50 mV – 409 mV difference spectrum, with peaks at 555 nm and 561 nm; this spectrum is essentially the fully reduced minus oxidized spectrum. In fig. 3B, the 164 mV – 409 mV difference spectrum (partly reduced minus oxidized) shows a selective decrease in the 555 nm peak corresponding to the range of redox potential in which the high-spin EPR signal increases. The 250 mV – 409 mV difference spectrum, shown in fig. 3C, consists primarily of a small amount of a 562 nm

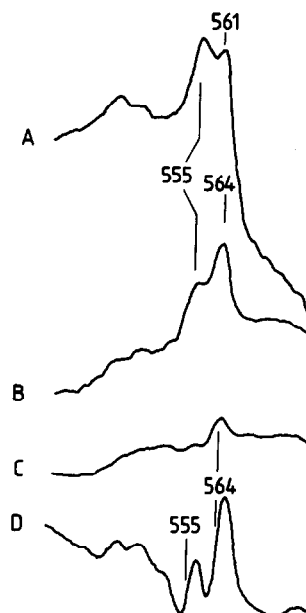


Fig. 3. Optical difference spectra at 77 K. Difference spectra between potentiometrically poised samples were obtained at 77 K using a split beam spectrophotometer. The samples were from the EPR redox titrations and were in the 3 mm EPR tubes. (A) 50 mV minus 409 mV; (B) 164 mV minus 409 mV; (C) 250 mV minus 409 mV; and (D) 164 mV minus 50 mV.

component, consistent with the partial reduction of the low-spin component.

Shown in fig.3D is a 164 mV – 50 mV difference spectrum (partly reduced minus reduced). The appearance of a positive peak at above 564 nm as well as the troughs at 555 and 559 nm cannot be explained in terms of oxidation of independent heme components. The peak to trough amplitude in the 'derivative-like' feature increases to a maximum as the reference (lower) potential is lowered; no such feature can be observed when the lower potential sample is poised at potentials greater than 220 mV. The amplitude of the same feature is greatest when the potential of the measuring (higher potential) sample is about 220 mV. When the potential of the measuring sample is much above 260 mV, only negative deflections can be observed corresponding to ferroheme oxidation. As the potential is lowered below 180 mV, the amplitude of the derivative-like feature decreased and is negligible below 100 mV. The difference between the two samples shown has about half-maximal intensity. Changes in the low temperature and room temperature (not shown) optical spectra between 250 mV and 420 mV suggest that no change in redox state of the hemes occurs, apart from the reduction of the remaining portion of the low-spin heme, that could account for the spectrum shown in fig.3D.

4. DISCUSSION

Previously reported potentiometric titrations of the cytochrome *bo* system, utilizing optical monitoring at ambient temperature and at 77 K (summarized in [1,2]), give a confused picture. Cytochrome *o* appears to titrate with an E_{m7} value of approx. 125 mV and cytochrome *b* with an E_{m7} value of approx. 200 mV (values averaged) [2,12–16]. Many of these redox titrations were conducted on strains with wild type expression levels where the phenotypes had to be one in which the expression of cytochrome *bo* was not maximal to avoid overlap with cytochrome *bd*. The results of our EPR monitored titrations on a strain lacking cytochrome *bd* confirm that at pH 7.0, high-spin cytochrome *o* titrates with a mid-point potential of approx. 160 mV while low-spin cytochrome *b* titrates with an E_{m7} value of approx. 250 mV.

EPR spectra of cytochrome *bo* have been

published; both high-spin and low-spin species, similar in *g* value to most reported here, were observed. The redox behavior of these components of the oxidase was not reported [8]. The most striking feature of the EPR monitored titrations is the fall in the intensity of the high-spin ferriheme EPR signals as the potential is raised above 300 mV. It is unlikely that the heme is either reduced in response to the oxidation of another group or further oxidized to the FeI state; the latter possibility would imply a gap of only 200 mV between the FeII/FeIII and FeI/FeII couples. No optical changes suggesting a change in heme redox state accompany the loss of the *g* = 6 EPR signal. A similar phenomenon, observed in the related cytochrome *aa*₃ system, is caused by tight spin coupling between the cytochrome *a*₃ heme and its associated copper (Cu_{a3}). Oxidation of cytochrome *a*₃ to the ferric state when the copper is reduced (CuI) results in the appearance of the *g* = 6 signal; oxidation of copper to CuII results in the loss of the EPR signal because coupling between copper (*S* = 1/2) and heme (*S* = 5/2) results in a net even spin system. Copper has been reported to be a constituent of the cytochrome *bo* system [12]. The loss of the *g* = 6.0 signal at high potential suggests that the catalytic site in this system, as in the cytochrome *aa*₃ system, consists of a closely coupled heme/copper pair. If this is so, an E_{m7} value for the CuII/CuI(o) of about 350 mV is indicated.

The low temperature optical spectra indicate that the 555 nm band is associated with the cytochrome *o* and that the 561 nm band is associated with the low-spin cytochrome *b*. The difference spectrum of fig.4D is of particular importance. As the potential is lowered from 409 mV using the oxidized spectrum as a baseline, positive peaks corresponding to the α -bands of ferrohemes appear. The observation of difference spectra with positive and negative deflections in the heme α -band region indicates that the α -band of one ferroheme shifts in response to reduction of another component. The fact that the derivative shaped feature can only be observed when the higher potential sample contains reduced cytochrome *b* and oxidized cytochrome *o* and the lower potential sample contains a significant level of reduced cytochrome *o* is indicative of heme/heme interaction. The simplest hypothesis is that reduction of cytochrome *o* shifts the α -band of fer-

cytochrome *b* from 564 nm to 561 nm (at 77 K), this can also be seen by comparing fig.3A and B. A heme-heme interaction is also a feature of cytochrome-*aa*₃ oxidase [7,17,18].

The striking similarities revealed between the cytochrome *bo* and cytochrome *aa*₃ systems should open the door to further physical and protein structural studies which will contribute to our understanding of both systems.

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