Heme-Copper and Heme-Heme Interactions in the Cytochrome bo-containing Quinol Oxidase of Escherichia coli*

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The cytochrome bo quinol oxidase of Escherichia coli is one of two respiratory O2 reductases which the bacterium synthesizes. The enzyme complex contains copper and 2 mol of b-type heme. Electron paramagnetic resonance (epr) spectroscopy of membranes from a strain having amplified levels of this enzyme complex reveals signals from low- and high-spin b-type hemes, but the copper, now established as a component of the oxidase, is not directly detectable by epr. The high-spin signal from the cytochrome bo complex, which we attribute to cytochrome o, when titrated potentiometrically, gives a bell-shaped curve. The low potential side of this curve is biphasic (Em7 approximately 180 and 280 mV) and corresponds to the reduction/oxidation of the cytochrome(s). The high potential side of the bell-shaped curve is monophasic (Em7 approximately 370 mV) and is proposed to be due to reduction/oxidation of a copper center which, when in the Cu(II) form, is tightly spin-coupled to a heme, probably cytochrome o, resulting in a net even spin system and loss of the epr signal. The spin coupled heme can be titrated biphasically with E m7 values of approximately 180 and 280 mV, similar to the high-spin component but without the loss of signal at high potentials.

Wild-type Escherichia coli is capable of synthesizing two different quinol oxidases; these enzymes constitute the terminal (O2-reducing) portion of the respiratory chain, performing the same role that cytochrome c oxidase does in mammalian mitochondria. The two E. coli oxidases, cytochrome bo and cytochrome bd, are differentially induced, apparently in part due to the O2 tension (for reviews see Refs. 1–3). The cytochrome bo complex is the dominant oxidase at high O2 tensions; the cytochrome bd predominates in cells grown at low aeration or under some anaerobic conditions. Of the four or five major classes of cytochrome oxidase found in bacteria, cytochrome o is the most widespread (2, 4). Cytochrome o (o for oxidase) was discovered in E. coli and in other bacteria by photochemical action spectroscopy of CO-inhibited respiration (6, 6). The CO and O2 binding pigment of the cytochrome bo complex is the o-moiety, and this is also a b-type cytochrome. Recently, genetic analysis of E. coli and the availability of strains lacking the cytochrome bd enzyme and having amplified expression of the cytochrome bo have made this oxidase much more amenable to experimentation. There are strong structural and biophysical similarities between the cytochrome bo and the mammalian cytochrome c oxidase, cytochrome aa3. Both the cytochrome aa3 and cytochrome bo contain copper and high- and low-spin hemes (7, 8). In the cytochrome oxidase aa3, the two hemes are of the a-type; in the latter they are of the b-type. As reported herein, the cytochrome bo, like the cytochrome aa3, exhibits both heme-heme and heme-copper interactions. The cytochrome bo complex contains four subunits (9, 10). DNA sequence analysis has revealed homology both with bacterial and mammalian cytochrome aa3. Subunit I of cytochrome bo has extensive regions of homology with the mammalian cytochrome aa3 subunit I. This is the subunit of cytochrome oxidase implicated in binding the hemes and one of the copper subunits (11, 12). One of the remaining subunits from cytochrome o is similar to subunit III of the cytochrome aa3 oxidase. There have been many investigations into the mechanism of action of mammalian cytochrome aa3, both from the H+-pumping aspect and regarding the O2 reduction mechanism (7). The realization that the cytochrome o system in E. coli is similar to the mammalian oxidase opens a possible route for enhancing progress on the mammalian system, by both a direct comparison and by exploiting the potential of the E. coli system for genetic manipulation.

In this paper we report potentiometric analyses of the cytochrome bo complex in a strain of E. coli that lacks cytochrome bd and has 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.

**MATERIALS AND METHODS**

E. coli strain RC145, which lacks the quinol oxidase cytochrome bo and has amplified expression of the cytochrome bo complex (13), was grown with aeration at 37 °C in 201 bottles. The medium contained (per 201): tryptophan, 200 g; yeast extract, 100 g; K2HPO4, 75 g; KH2PO4, 25 g; ampicillin, 2 g; thiamine, 2 g; nicotinic acid, 2 g; 50 μM Mo/Se supplement; and 20 ml of a trace metal solution (14). The medium was as normally prepared also contained a supplement of 50 mg CuSO4/201; this was omitted when copper-unsupplemented cells were required. Cells were harvested in the late exponential phase of growth, and cytoplasmic membranes particles were isolated by differential centrifugation following French Pressure cell treatment and stored as described previously (15).

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Fig. 1. Electron paramagnetic resonance spectra. Samples of RG145 membranes from a potentiometric redox titration at pH 7.0 were poised at 420 mV (top), 280 mV, 140 mV, and 100 mV (bottom) as described under "Materials and Methods." The g-values of the axial high-spin cytochrome o (g = 6.0) and the low-spin cytochrome b, g\textsubscript{o} = 3.00 and g\textsubscript{b} = 2.2 are indicated. The large signal between the g = 6.0 and g = 3.00 is due to low symmetry Fe(II) (E\textsubscript{m} = +217 mV), tetramethvl-n-phenvlenediamine (50 PM, E\textsubscript{m} = +340 mV), and ascorbate. In addition ferricyanide (E\textsubscript{m} = +420 mV) was titrated as oxidant and dithionite was used as reductant. Data from redox titrations at pH 6-9 are shown in Fig. 3; the curves drawn for the low-spin component (lower plot in each quarter) are the sum of two Nernst curves, and the curves drawn for the high-spin component (upper traces) are the best fits resulting from a fractional multiple of a summation of the two cytochrome (Nernst) curves with the copper curve (A, pH = 280 mV and declines as the potential is further increased to 420 mV (top trace). The low-spin ferricytochrome b signals at g = 3.00 and g = 2.2 increase as the potential is raised throughout this potential range and show no decline at high potentials. As the potential is altered the lineshape of the g = 3 signal and 2.2 features change as the complex goes partly reduced.

Fig. 2 shows plots of the intensities of the high-spin (a) and low-spin (b) signals against the potential of the poised sample; data are shown for pH 5.5. The membrane bound complex is stable at this pH and the resolution of the phases in the titration (high-spin) is better seen due to wider separation of the "copper" and cytochrome midpoint potentials. The titration of the low-spin heme, monitored at g = 3.00 and g = 2.9, is not well described as a single component with equivalence (n) of 1.0. The data have been fitted using two n = 1 components with midpotential values of 320 and 230 mV representing 36 and 64%, respectively, of the observable signal height. The titration of the g = 6 signal is more complex. The Nernst curves in Fig. 2a were drawn to represent two phases of cytochrome oxidation and one reverse phase attributed to Cu(I) oxidation. The rise of the g = 6 signal as the potential is raised to a peak can be modeled using two n = 1 components with potentials of 320 and 230 mV and respective contributions of approximately 64 and 36%, these are the same mid-point potentials as those observed for titration of the low-spin component, but the relative proportions are reversed. The fall of the high-spin signal at high potentials has been modeled as au n = 1 component. The accuracy of the estimation of the E\textsubscript{m} of this component, which probably represents a copper center closely coupled to cytochrome o, is limited by the lack of a good endpoint (except at alkaline pH), since we were unable to obtain potentials much above 420 mV with reasonable additions of ferricyanide. Data from redox titrations at pH 6-9 are shown in Fig. 3; the curves drawn for the low-spin component (lower plot in each quarter) are the sum of two Nernst curves, and the curves drawn for the high-spin component (upper traces) are the best fits resulting from a fractional multiple of a summation of the two cytochrome (Nernst) curves with the copper curve (A, pH =

RESULTS

Fig. 1 shows epr spectra at 15 K of cytoplasmic membrane particles of E. coli strain RG145 poised at different potentials at pH 7.0. The ambient potential (millivolts) prior to freeze-trapping is indicated at the left of each trace. The axial epr signal at g = 6 is small in magnitude at 100 mV (bottom trace); the size of the signal increases as the potential is raised to

2 The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tricine, N-tris(hydroxymethyl)methylglycine; Bicine, N, N-bis(2-hydroxyethyl)glycine.

FIG. 2. Plot of redox titration data of high- and low-spin centers. The redox titration was carried out at pH 5.5 as described under "Materials and Methods." The signal heights of the g = 6.0 signal (a, O-O) and the g = 3.00 and g = 2.2 signals (b, O-O, ▲-▲, respectively) are plotted against E\textsubscript{m}. Each curve represents a single redox phase of an n = 1 component. In a two Nernst n = 1 curves are fitted for the cytochrome o transition and one for the putative Cu(I)/II transition. In b to Nernst n = 1 curves are fitted for the cytochrome b transition. The same mid-point potentials are used to fit the cytochrome o and the cytochrome b.
FIG. 3. The effect of pH on potentiometric redox titrations. The plots of redox titrations for pH 6.0 (a), 7.0 (b), 8.0 (c), and 9.0 (d) are shown. In each quarter the upper trace shows the behavior of the high-spin component, and the lower trace shows the low-spin component. For the high-spin center, the curves drawn represent the best fit to three component $n = 1$ curves, two positive (representing the oxidized high-spin signal) and one negative curve (representing the Cu(I) center to which it is coupled) ($\circ$-$\bullet$). For the low-spin center the curve drawn represents the best fit to two positive $n = 1$ curves, $g = 2.2$ ($\Delta$-$\Delta$), $g = 3.00$ ($\bullet$-$\bullet$). The abscissae represent redox potential in millivolts. The ordinates are signal height. In the plots of the high-spin signal the scale is the percent of the calculated maximal signal height in the absence of the putative copper interaction, in the plots of the low-spin signal, the scale is arbitrary. The buffers and mediators used are listed under “Materials and Methods.”

Fig. 4 (a and b) shows the effect of CO on the potentiometric behavior of the high- (a) and low-spin (b) components (closed symbols); data obtained in the absence of CO is included for comparison (open symbols). Most of the high-spin signal is abolished in the presence of CO even at high potentials, the residual signal is a mixture of rhombic and axial components and titrates at approximately 150 mV. In the presence of CO the low-spin signal appears to titrate as a single component with an apparent midpoint of approximately +165 mV. In Fig. 4c spectra are shown of the low-spin $g$ signals of oxidized samples in the presence and absence of CO. The peak position of the low-spin $g = 3.0$ resonance shifts from 3.00 to 2.98, line-shape changes can also be observed at $g = 2.2$ (not shown).

A plot of the intensity of the $g = 6$ signals of membrane particles prepared from the cytochrome bo-overexpressing strain grown in a medium from which copper was omitted is shown in Fig. 5. The data are taken from a potentiometric titration performed at pH 7.0. The fall in the signal height at high potentials is observable but much less extensive than in the corresponding titration of membranes from copper-sufficient cells (Figs. 2a and 3). Also shown in Fig. 5 is the behavior of the high-spin center when titrated under a CO atmosphere.

FIG. 5. Redox titration of the high-spin signal of membranes from copper-unsupplemented cells. The signal heights of the $g = 6.0$ signal are plotted against potential under N$_2$ (open circles) and under CO (closed circles) atmospheres. The redox titrations were performed at pH 7 as described under “Materials and Methods.” Each curve represents a single $n = 1$ redox phase.

in a similar titration of copper-supplemented membranes (Fig. 4), only a small residual high-spin signal is observed throughout the potential range. These findings indicate that the component that is responsible for the loss of the signal is copper and implies that copper-deficient oxidase is synthesized and is reasonably stable in growing cells. The titration in the presence of CO has approximately the same end point at high potential as in the absence of CO, indicating that the small drop at high potential is due to the residual Cu heme center and that the major component in the CO titration is
the cytochrome o modified by the absence of copper. In the copper-supplemented case CO shifts the apparent midpoint potential of the high-spin species to approximately 350 mV from approximately 150 mV. The copper-deficient derivative of the oxidase appears to lack normal levels of activity; in minimal media (14 plus glycerol) a copper dependence for growth is indicated2 in the complex (nondefined) media used herein the ubiquinol-1 oxidase activity was 510 mol (UQH₂) · s⁻¹ · mol cytochrome o⁻¹ in the copper-supplemented membranes and 280 mol · s⁻¹ · cytochrome o⁻¹ in the copper-deficient membranes. The oxidation of ubiquinol-1 was measured at 278 nm according to the method of Kita et al. (18). This ratio of activities is comparable to the ratio of contributions from the copper-deficient and sufficient forms of the enzyme synthesized in the copper-deficient media (Fig. 5), i.e. approximately 50% of the cytochrome o synthesized under these copper-limited conditions lacks copper and has no or low activity.

The biphasic titration of the cytochromes in the copper-sufficient case can be modeled in terms of heme-heme cooperativity. A diagram of a cooperative model is shown in Fig. 6. The model is simplified by the omission of the copper redox changes. The four states represent the four possible redox conditions of the heme pair. E₀ and E₅ are the midpoint potentials of each heme when the other is oxidized and Δ represents the strength of the cooperative interaction. Analysis of the proportions of cytochrome o and b reduced in the high-potential phase and the midpoint potential of the phase gives the values for E₀ and E₅. Negative values of Δ (in millivolts) correspond to anti-cooperative behavior in which reduction of one heme lowers the potential of the other.

Analysis of titration data at various pH values between 5.5 and 9.0, in terms of this model, produced values for E₀, E₅, and Δ for the hemes as a function of pH. The midpoint potential of the copper was estimated from the decrease of the g = 6 signal at high potential. These parameters are plotted against pH in Fig. 7. The cooperative interaction is insensitive to pH and its value is approximately −60 mV. The midpoint potentials of the two hemes are close; at neutral pH the potential of the low-spin cytochrome b is slightly (10–20 mV) higher than the cytochrome o so that approximately 60% of the cytochrome b and 40% of the cytochrome o titrate at the higher potential and conversely 60% of the cytochrome o and 40% of the cytochrome b titrate at the lower potential. The redox behavior of both hemes appear weakly pH-dependent. At pH 5.5 the potential of the high-spin cytochrome o is apparently slightly higher than that of the low-spin cytochrome b, which causes it to be the major constituent of the high potential component. An overall pattern of weak pH dependence (average 10–20 mV/pH unit) is indicated for the two hemes. This falls short of the 59 mV · pH⁻¹ unit expected for pH dependence (one H⁺/electron) redox reaction but is a feature shared with the hemes of cytochrome c oxidase. Thus pKₐ values cannot be assigned. The copper center shows a greater pH dependence above pH 8.0, and a functional pKₐ may exist, but the data are not strong as the onset of the dependence is close to the limit of the pH range over which the data are obtained.

**DISCUSSION**

In this work a strain of *E. coli* was used which had amplified levels of expression of cytochrome bo. The use of this strain is justified from a comparison of data from strains with amplified and unamplified expression levels. These show that the specific activities of the enzyme are not significantly modified by overexpression. The enzyme complex has a similar composition when isolated from either source (four polypeptides M, approximately 66,000, 55,000, 22,000, and 17,000) (9)). The activity of the ubiquinol-1 oxidase parallels the expression level of the oxidase in the membrane (13), and the biophysical parameters reported herein are similar to those found in membranes from unamplified strains (19).

Carbon monoxide binding appears to alter both the high-spin and the low-spin signals of the cytochrome bo, although the major change is clearly to the high-spin signal (Fig. 4), confirming that the high-spin species is also the oxygen-binding species, by definition, the heme o. Hata et al. (8) found that cyanide, which binds to the oxidized rather than the reduced form, also distorts the high-spin signal. Because of the heme cooperativity that we observe in this system, one would expect the redox behavior of the low-spin component also to be affected, as seen in Fig. 4b. The effect of CO on the midpoint potential of the low-spin component is slightly larger than predicted on the basis of cooperativity alone; this would predict that the low-spin component titrated as a single component with an apparent midpoint of +230 mV (pH 7.0). Reid and Ingledew (20) using optical spectroscopy found a single b-component titrating at +190 mV in the presence of

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1 M. Bacon, B. Bolgiano, R. K. Poole, and W. J. Ingledew, unpublished observations.

2 K. C. Minghetti and R. B. Gennis, manuscript in preparation.
CO. Judging from our results with the copper-unsupplemented cells (Fig. 5), the copper associated with the heme o appears to exert a strong effect on the strength of CO binding to that heme.

The evidence for a role for copper in cytochrome bo comes from analyses which show its presence in the purified complex (6, 9), the copper dependence of the oxidase activity, and the explanation of the bell-shaped titration data for the high-spin heme signal. Our results indicate that the catalytic site of cytochrome bo is likely to be a tightly coupled heme-copper binuclear center, very similar to that present in the cytochrome aa oxidase (21, 22). The loss of the g = 6 epr signal on oxidation of a center (Figs. 1 and 2) is reminiscent of the mammalian and bacterial cytochrome aa systems and implies a magnetic interaction comparable with, or stronger than, the Zeeman interaction (approximately 0.3 cm\(^{-1}\)) in fields appropriate for X-band epr. In the cytochrome aa system, a heme-copper distance of less than 5 Å produces coupling at least an order of magnitude stronger than the Zeeman term (23). Other evidence that the high-spin cytochrome o is coupled with Cu(II) comes from the results obtained with copper-deficient membranes (Fig. 5). Although copper epr signals were observable in some of our preparations (data not shown), they were substoichiometric compared with cytochrome bo and probably represent adventitious copper. The CuII associated with the binuclear center (designated Cu(II), to indicate its association) would not be epr-detectable under normal circumstances. Cytochrome bo probably lacks a center analogous to the epr-detectable copper (Cu(II)) of mammalian cytochrome c oxidase. Center "Cu", analogous to the CuII of the cytochrome c oxidase binuclear site (Cu(II)) has an apparent potential about 100 mV more positive than either heme.

A heme-heme interaction is indicated in cytochrome bo by the changes in the oxidized (as shown by epr) and reduced (as shown by optical spectroscopy) forms of the cytochrome b when cytochrome o undergoes redox changes (18, Fig. 4c). The titrations of both hemes are multiphasic but this behavior can be satisfactorily modeled in terms of heme-heme cooperativity. This is again reminiscent of the cytochrome aa system (21), although the interaction strength required is slightly smaller than that proposed for the coupling between hemes a and a (60 versus 87 mV, respectively). The heme-heme interaction model has the additional virtue of explaining differences in relative potentials (Refs. 20, 24-27, reviewed in Ref. 2). Attempts to deconvolute the optical spectra in the o-regions are reviewed by Poole and Ingledew (2). Recently, using epr spectroscopy on a strain in which cytochrome bd has been deleted, but which does not have amplified expression of cytochrome bo, we have observed at least 80% of the b and o-centers titrating at 250 and 160 mV, respectively (19). If cooperativity between the two hemes is the reason for the biphasic titrations of both hemes reported herein, a small shift in the relative potential of the two hemes would account for the discrepancy. In terms of our model (Fig. 5), E\(_o\) = E\(_b\) would give biphasic 50:50 titrations of both components, and the two hemes would be inseparable, whereas the E\(_o\) = E\(_b\) = 60 mV would allow 90% of the cytochrome bo to titrate at high potential while 90% of the cytochrome o titrated at low potential. It is probable that a 20-30 mV shift in the relative potentials of the two hemes would account for the discrepancies in the literature. Shifts of this magnitude depending on lipid composition, ionic strength, and the content and nature of mediators used in titrations are not unlikely.

The shifts in the optical (18) and low spin (9) epr spectra of b-heme during titration of cytochrome bo also provide support for a heme-heme interaction. The small shift in the epr spectra can be interpreted in terms of a modest change in the geometry of the low-spin d-site in response to oxidation/reduction of the high-spin o-heme. In the fully oxidized complex a crystal field calculation gives $\Delta$ = 3.2 $\lambda$, V = 1.62 $\lambda$ which implies bis-histidine ligation with only slight strain compared with unhindered bis-imidazole model complexes (26).

The behavior of the hemes and the catalytic site copper of cytochrome bo appears to be qualitatively similar to the corresponding groups in the mammalian cytochrome aa system. The midpoint potentials are slightly lower in the cytochrome bo system, which may reflect the greater reducing power of the ubiquinol donor (E\(_0\) = 60 mV) compared with cytochrome c (E\(_0\) = 240 mV). The apparent lack of a Cu(II) analog probably also reflects the different donor. Cu(II) is probably the acceptor from cytochrome c in the cytochrome aa system, and ubiquinol may be able to donate electrons more directly, since its binding site is likely to be within the bilayer-spanning hydrophobic region.

In conclusion the catalytic site of the cytochrome bo quinol oxidase appears to be a binuclear heme-copper center similar to that of cytochrome aa. The low spin cytochrome bo appears closely analogous to cytochrome o in that system. Heme-heme interactions are also observed in both systems. The striking similarities between subunit I of the cytochrome aa system and subunit I of the cytochrome bo system promise to contribute greatly to our understanding of both systems.

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