Midpoint Potentials of the Mitochondrial Cytochromes of
Crithidia fasciculata

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The midpoint potentials of the mitochondrial respiratory chain cytochromes of the protozoan Crithidia fasciculata at pH 7.2, Eₘ₇.₂, show great similarity to those measured in higher organisms. Values of Eₘ₇.₂ for cytochromes a and a₃ are +165 and +340 mV. Both c cytochromes have Eₘ₇.₂ = +230 mV. There are two b cytochromes with the same spectral characteristics with Eₘ₇.₂ = −20 and −135 mV. These values are compatible with two sites of energy conservation for oxidative phosphorylation in these mitochondria. All cytochrome components show potentiometric titrations with n = 1. There is a fluorescent flavoprotein in these mitochondria with Eₘ₇.₂ = −40 mV and n = 2, whose function is not known.

In studies extending over the past several years, the presence of hemoprotein electron transport carriers in the mitochondrial membrane of the trypanosomatid Crithidia fasciculata has been demonstrated (6, 9, 11; J. P. Kusel and M. M. Weber, Bacteriol. Proc. p. 139, 1968), and the similarity of the mitochondrial respiratory chain of this protozoan to that of mammalian and avian species has been noted (9, 10). Mitochondria isolated from C. fasciculata have cytochrome a + a₃ as the only functional terminal oxidase (6) and have, in addition to salt-extractable cytochrome c-555 analogous to mammalian cytochrome c (11), a type c cytochrome tightly bound to the membrane analogous to mammalian cytochrome c (9).

Difference spectra obtained at 77°K show but one resolvable absorbance maximum attributable to the type b cytochromes (9). Full characterization of mitochondrial electron transport carriers with regard to function and placement in the respiratory chain requires determination of the midpoint potentials, however (4), since this determination differentiates between species with nearly superimposable absorbance maxima. In this paper, we report the values of the midpoint potentials of the mitochondrial cytochromes of C. fasciculata and the differentiation of the cytochrome b component into two separate species.

MATERIALS AND METHODS

Cell culture. C. fasciculata (ATCC 11745) was cultivated in the undefined medium of Kidder and Dutta (7) with the following modifications: (i) glycerol at 0.11 M replaced glucose as the carbohydrate energy source; (ii) crystalline triethanolamine hydrochloride was used at 0.08 M; (iii) Tween 80 (polyisorbitan monoooleate) was omitted; (iv) the complete medium at pH 8.0 minus hemin was transferred in 950-ml portions to 2-liter Erlenmeyer growth flasks and steam sterilized at 121°C for 25 min; (v) hemin stock solution was prepared separately at 0.75 mM in 0.1 M KOH adjusted to pH 8.5 with 1.0 M triethanolamine hydrochloride, sterilized at 121°C for 15 min, and stored at −20°C in the dark. It was added aseptically to the growth medium immediately prior to cell transfer at a final concentration of 7.5 μM.

Cultures were grown at 28 to 30°C in New Brunswick gyratory shakers at 50 cycles/min. The doubling time was 4.5 h in the logarithmic growth phase, which persisted until culture densities of 2.0 × 10⁶ to 2.5 × 10⁸ organisms/cm² were attained. Cells were collected in the late logarithmic phase with a Sharples continuous-flow centrifuge and resuspended in 10 volumes of buffer containing 0.3 M mannitol and 5 mM morpholinopropanesulfonic acid (MOPS) adjusted to pH 7.5 with KOH.

Preparation of mitochondria. Washed cells were resuspended to 5% (wt/vol) in ice-cold isolation medium containing 0.3 M mannitol, 5 mM MOPS, 2.0 g of polyvinylpyrrolidone per liter, 3.0 g of defatted bovine serum albumin (fraction V, Nutritional Biochemicals Corp.) per liter, 4.5 mM ascorbic acid, 250 μM MgCl₂, and 250 μM ethylenediaminetetraacetic acid at pH 8.0 adjusted with KOH. Digitonin in dimethylformamide (40 mg/ml) was added dropwise to the cell suspension with rapid stirring to provide 6.0 mg/g of cells. After slow stirring for 30 to 40 min at 0 to 4°C, the cells were recovered by centrifugation at 1,500 × g for 10 min and were suspended to 10% (wt/vol) in isolation medium. Cells were disrupted with a Polytron (Bronwill Scientific Co.), using one 30-s treatment at maximum speed with the addition of an equal volume of isolation medium.
after 20 s; lysis was 75 to 90%. Unbroken cells and debris were removed by centrifugation at 680 × g for 10 min. Centrifugation of the supernatant fluid at 12,000 × g for 10 min yielded a crude mitochondrial pellet that was suspended with a glass-Teflon homogenizer in wash medium, using approximately 1 ml of wash medium per 5 ml of crude homogenate. Wash medium was identical with isolation medium but without ascorbate. Centrifugation at 12,000 × g for 10 min yielded a supernatant fluid and upper layer that were removed by aspiration and a brown mitochondrial layer and underlying viscous white button that were suspended in half the previous volume of wash medium. Particulate aggregates were removed by centrifugation at 480 × g for 5 min. The supernatant fluid was then removed, and the brown mitochondrial layer was resuspended in one-fourth volume of wash fluid used in the first wash step, avoiding the viscous white underlayer. The last two centrifugation steps were repeated, and the mitochondria were suspended in a minimal volume of wash medium.

Freshly prepared mitochondria contained too much endogenous substrate for accurate and reversible measurement of oxidation-reduction potentials. The endogenous substrate was removed by the following hypotonic treatment. Fresh mitochondrial preparations, suspended in 0.3 M mannitol-5 mM MOPS, pH 7.5, at 60 to 80 mg of protein per ml, were diluted by slow, dropwise addition with continuous stirring of 18 volumes of 10 mM MOPS containing 5 mM potassium phosphate, pH 7.2. Mitochondria were sedimented by centrifuging for 10 min at 12,000 × g and were suspended in the same hypotonic buffer to a protein concentration of 3 to 4 mg/ml. These were kept overnight at 0 to 4°C in ice and then centrifuged as above and washed once by suspension in hypotonic buffer. After centrifugation, the pellet was resuspended in a minimal volume of hypotonic buffer, and 18 volumes of 0.3 M mannitol buffered with 10 mM MOPS, pH 7.2, were added dropwise with continuous stirring. This buffer was used to wash the mitochondrial fraction twice to remove phosphate and to suspend the final pellet. This, in turn, was diluted as required with 0.3 M mannitol-10 mM MOPS, pH 7.2, for the potentiometric measurement.

Redox potential measurements. Redox potential measurements were carried out at 22 to 24°C, using the method described in detail by Wilson and Dutton (14), in which the titration can be carried out in the presence of argon with or without carbon monoxide. A dual wavelength spectrophotometer compensated for light source noise (9) was used to measure the extent of cytochrome or flavoprotein reduction, and a Radiometer potentiometer equipped with P101 platinum and K401 reference electrodes was used to measure the potential of the suspension reported by cytochrome-reduction mediator dyes added for this purpose (5). For the spectrophotometric measurement, a measuring wavelength of 562 nm and reference wavelength of 544 nm were chosen to monitor the absorbance changes due to oxidation or reduction of both type b and c cytochromes, since 562 nm was found to give an absorbance change with redox state at room temperature (20 to 22°C) with both c cytochromes as well as the b cytochromes (9). The reference wavelength is near the 545-nm isosbestic point determined for purified Crithidia fasciculata cytochrome c-555 (11). The wavelengths 445 and 455 nm, respectively, were used to measure absorbance changes due to cytochromes a + a3. The wavelengths 468 and 493 nm were used for the simultaneous measurement of flavoprotein absorbance and fluorescence (13). Absorbance changes were corrected for absorbance changes due to the oxidation-reduction dyes employed. Multiple components were resolved mathematically, as described by Dutton and Wilson (4). Replicate experiments showed the midpoint potential determinations to be reproducible to within ±10 mV.

RESULTS

Cytochromes a and a3. The potentiometric titration of the cytochromes of C. fasciculata mitochondria at the wavelengths 445 to 455 nm, suitable for monitoring cytochromes a + a3, is shown in Fig. 1A. The complex curve can be resolved into three separate species (Fig. 1B), with midpoint potentials $E_{m7.2}$ of +340 mV, +165 mV, and +5 mV with $n = 1$ as expected for cytochrome hemoproteins. The two high-potential cytochromes comprise 86% of the total absorbance change at this wavelength pair. In the presence of CO, which binds to reduced cytochrome a3 to give a complex with midpoint potential too positive to be measured by this method (4), the absorbance change for the +340-mV component is reduced by 68%, but the absorbance change for the other two compo-

![Fig. 1. Potentiometric titration of the cytochrome a + a3 components of C. fasciculata mitochondria suspended in 0.3 M mannitol-10 mM MOPS, pH 7.2, at 2.5 mg of protein per ml. Redox mediators were: phenazine methosul fate, 2 μM; phenazine ethosul fate, 2 μM; N,N'-tetramethylphenylenediamine, 80 μM; K3Fe(CN)6, 160 μM. The titration range was +390 to −100 mV. The absorbance change was corrected for mediator contributions with a blank titration. (A) Total absorbance change as a function of potential; (B) resolution into three components with $n = 1$.](http://jb.asm.org/)
nts remains essentially unchanged. The high-potential component with \( E_{m7.2} = +340 \) mV can therefore be attributed to cytochrome \( a_3 \) and that with \( E_{m7.2} = +165 \) mV can be attributed to cytochrome \( a \). At this wavelength pair, the CO-binding pigments, which are present in these mitochondria but not part of the respiratory chain, give no absorbance change (10). In the absence of CO, the ratio of the absorbance change for cytochrome \( a_3 \) to that for cytochrome \( a \) is 0.84. The low-potential component is attributed to interference from the Soret band of the \( b \) cytochromes. Since the redox titration was designed to determine the values of \( E_{m7.2} \) for cytochromes \( a + a_3 \), it was not carried to values of \( E_b \) sufficiently negative to give complete reduction of the cytochrome \( b \) component. The value \( E_{m7.2} = +5 \) mV must, therefore, be considered an estimate that errs to a more positive value.

**Cytochromes \( c \).** The complex potentiometric titration curve obtained at the wavelength pair 562 to 544 nm (Fig. 2A) can be resolved into four components (Fig. 2B). The high-potential component at \( +230 \) mV can be attributed to cytochrome \( c-555 \) (11) plus cytochrome \( c_1 \) (9), by analogy with other mitochondria (3, 5). The other three components are candidates for type \( b \) cytochromes.

**Cytochromes \( b \).** If the potentiometric titration is repeated in the presence of CO, there is a reduction of 45% in the absorbance change due to the component with \( E_{m7.2} = +100 \) mV but no change in midpoint potential and a 50% increase in absorbance change, with a shift in midpoint potential to \( E_{m7.2} = 0 \) mV of the component originally titrated at \( E_{m7.2} = -20 \) mV. Since previous work (10) has shown that spectral changes due to CO-binding pigments not associated with the respiratory chain should be observed at this wavelength pair, we attribute these effects to interference by these pigments. The midpoint potential of the CO-ligated form of a hemoprotein is always more positive than that of the unliganded form (4). The 45% loss of absorbance of the component with \( E_{m7.2} = +100 \) mV is, therefore, attributed to a CO-ligated form, with \( E_{m7.2} \) too positive to be seen in these titrations, similar to the 66% loss of absorbance observed with cytochrome \( a_3 \). The component with \( E_{m7.2} = +100 \) mV can be identified with the CO-binding pigment readily reducible by succinate but not part of the respiratory chain (10). The apparent increase in absorbance and shift in \( E_{m7.2} \) of the component originally titrated at \( E_{m7.2} = -20 \) mV is attributed to interference from one of the mitochondrial CO-binding pigments reducible only by dithionite (10), whose \( E_{m7.2} \) in the unliganded form is too negative to be found in these titrations but which shifts to around 0 mV on binding CO, so that it and the component with \( E_{m7.2} = -20 \) mV titrate as a single, unresolved pigment with an absorbance change given by the sum of both components. Since there is no loss in absorbance of the component with \( E_{m7.2} = -20 \) mV, this component can be identified as a \( b \) cytochrome. There is no effect of CO on the component with \( E_{m7.2} = -135 \) mV, showing that it is also a \( b \) cytochrome. The cytochromes of the mitochondria of *C. fasciculata* are listed with their midpoint potentials in Table 1.

Attempts to show spectral differences between the \( b \) cytochromes over the wavelength range 560 to 566 nm were unsuccessful, implying that both cytochromes have a characteristic absorbance band at 77°C in the reduced state at

**Table 1. Midpoint potentials at pH 7.2, \( E_{m7.2} \), of the mitochondrial cytochromes of *C. fasciculata*.

<table>
<thead>
<tr>
<th>Cytochrome</th>
<th>( E_{m7.2} ) (mV)</th>
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<tbody>
<tr>
<td>( a_3 )</td>
<td>+340</td>
</tr>
<tr>
<td>( a )</td>
<td>+165</td>
</tr>
<tr>
<td>( c-555 )</td>
<td>+230</td>
</tr>
<tr>
<td>( c_1 )</td>
<td>+230</td>
</tr>
<tr>
<td>( b_m )</td>
<td>-20</td>
</tr>
<tr>
<td>( b_L )</td>
<td>-135</td>
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* The subscripts M and L indicate middle- and low-potential cytochromes \( b \).
557 nm and at 561 nm at room temperature, as inferred from previous studies (9). No apparent shift in $E_{m7.2}$ of the cytochrome $b$ with low midpoint potential, designated cytochrome $b_1$, to more positive values induced by adenosine triphosphate could be observed (14).

**Flavoproteins.** Determination of the midpoint potentials of the flavoproteins of *C. fasciculata* mitochondria by potentiometric titration based on absorbance changes was thwarted by the presence of too many components for successful resolution of individual components. One flavoprotein was fluorescent and gave a straightforward redox titration yielding $E_{m7.2} = -40$ mV with $n = 2$. Its role in the respiratory chain has not been established.

**DISCUSSION**

The conclusion drawn from previous spectroscopic studies at 77°C (9), namely, that the respiratory chain of *C. fasciculata* mitochondria is remarkably similar to that of mitochondria from higher animals and plants, is strongly supported by the potentiometric study reported here. The $E_{m7.2}$ values for cytochromes $a$ and $a_3$ are 40 mV more negative than the values generally assigned to these cytochromes in mammalian mitochondria (4) but are within the range found in various mitochondrial preparations and submitochondrial particles (5) and are also similar to the values obtained for these cytochromes in plant mitochondria (3). Further, the difference between the midpoint potentials is the same as found in other mitochondria and, therefore, consistent with an energy conservation site. The value of +230 mV for the c cytochromes is identical to that found for these cytochromes in mitochondria isolated from both animal and plant tissues (3, 5, 6). It is also consistent with the value of $E_{m7.0} = +280$ mV reported for cytochrome c-555 in soluble form (11); Dutton et al. (5) have shown that horse heart cytochrome $c$ has a midpoint potential over the pH range 7.0 to 7.2 of 270 to 280 mV, depending on buffer type, which changes to +230 mV when the cytochrome $c$ is bound to phospholipid vesicles or to submitochondrial particles.

Some divergence from similarity to mammalian mitochondria is shown by these protozoan mitochondria with regard to the cytochrome $b$ component in that the midpoint potentials are lower than expected. Two type $b$ cytochromes are found, however, as reported originally for rat liver and pigeon heart mitochondria (12, 14). It seems reasonable to consider that cytochrome $b_M$ with $E_{m7.2} = -20$ mV and cytochrome $b_t$ with $E_{m7.2} = -135$ mV in *C. fasciculata* correspond to cytochrome $b_K$ and $b_T$, respectively, in animal mitochondria (12) and to cytochromes $b-560$ and $b-565$ in plant mitochondria (3). Cytochrome $b_1$ is about 110 mV more negative than cytochrome $b_K$; this is a larger difference than is found in animal mitochondria (14) but is about the same as that found in plant mitochondria (3). It appears that *C. fasciculata* contain two type $b$ cytochromes similar to cytochromes $b_K$ and $b_T$ but with absolute values of $E_{m7.2}$ shifted to more negative values. The more negative midpoint potentials of the $b$ cytochromes result in better matching to the midpoint potential of a preferred oxidative substrate of these mitochondria, 1-L-glycerol phosphate (8); $E_{m7.0}$ for the L-1-glycerol phosphate-dihydroxyacetone phosphate couple is $-192$ mV (1). They are also consistent with the location of the two sites of energy conservation in these mitochondria (8). The great similarity between the respiratory chains of the protozoan mitochondria and of those of much more highly evolved organisms underscores the extraordinary evolutionary success of this method of aerobic energy abstraction from metabolites.

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**LITERATURE CITED**


