The Mitochondrial b-Cytochromes of the Wild Type and Poky Strains of Neurospora crassa

EVIDENCE FOR A COMPONENT REDUCED ONLY BY DITHIONITE*

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SUMMARY

Mitochondria isolated from the wild type and poky strains of Neurospora crassa are shown to contain three b-cytochromes. Two of these—b-561 and b-566 (b-558)—are reduced by physiological substrates, whereas the third (a-peak (25°C) at 558 nm) is reduced only by dithionite. The concentration of all three b-cytochromes is much lower in poky than in wild type mitochondria, but the ratio of b-561 to b-566 (b-558) remains 1:1 in both strains. On the other hand, the proportion of "dithionite-reducible" b, measured by potentiometric titrations at wavelength pair 559-540 nm, increases from 30% in wild type to 55 to 60% in poky. The presence of the dithionite-reducible component has been overlooked by other workers leading to an apparently erroneous report that the stoichiometry of b-561 and b-566 (b-558) is 6:1 in poky mitochondria (von Jagow, G., Wass, H., and Klingenberg, M. (1973) Eur. J. Biochem. 33, 140). The measured midpoint potentials of b-561, b-566 (b-558), and dithionite-reducible b in wild type mitochondria are 78 ± 2, -73 ± 8, and -166 ± 4 mv, respectively. Roughly the same midpoint potentials are found for the b-cytochromes of poky and the results support the idea that, except for concentration, the b-cytochromes are not significantly altered by the poky mutation.

Poky (mi-1) is a cytoplasmically inherited mutant of Neurospora crassa characterized by a slow growth rate, a defect in mitochondrial ribosomal assembly, and deficiencies in several cytochromes (1-4). Most (about two-thirds) of poky's respiration is mediated by a novel hydroxamic acid-sensitive oxidase system which is resistant to the conventional respiratory inhibitors (cyanide and antimycin) and which is not present constitutively in wild type (5-7). The remaining one-third of poky's respiration is mediated by the residual cytochrome system. The components of this system are qualitatively similar to those of wild type mitochondria, but with the exception of cytochrome c, they are present at much lower concentrations (2-4, 7). Even so, since the alternate oxidase is nonphosphorylating, the cytochrome system accounts for nearly all of the oxidative phosphorylation in log phase poky cells (6). One matter of controversy that has arisen regarding the poky cytochrome system concerns the number and stoichiometry of the b-cytochromes. On the basis of studies by Lambowitz et al. (4) and von Jagow et al. (7), it is agreed that Neurospora mitochondria contain at least two b-cytochromes, b-561 and b-566 (b-558), analogous to components found in animal and higher plant mitochondria, and that these two b-cytochromes are present in equal concentration in the wild type strain judged from low temperature spectra of aerobic, antimycin-treated mitochondria reduced with succinate (conditions for which the b-cytochromes are maximally reduced; see Fig. 1 of Ref. 4). For poky, on the other hand, von Jagow et al., estimating concentrations from spectra of dithionite-reduced mitochondria, reported that the concentration of b-561 was six times that of b-566 (b-558), while in the study of Lambowitz et al., with reduction by succinate plus antimycin, it was estimated that the b-cytochromes were present in equal concentrations (see Fig. 3 of Ref. 4). The result of von Jagow et al. was purported to demonstrate that the stoichiometry of the b-cy complex is variable and might also suggest that incorporation of the two b-cytochromes into the mitochondrial membrane is independently regulated. However, the discrepancy is more prosaically explained by the possibility suggested by Lambowitz et al. that

1 Following the recommendation of the IUB Cytochrome Nomenclature Subcommittee, b-561 and b-566 are named according to the positions of their a-peaks in reduced minus oxidized difference spectra at room temperature. In previous work on Neurospora mitochondria, these components had been designated b561 and b566 according to their a absorption maxima at 77K (Ref. 4) and b561 and b566 by analogy with the nomenclature advanced by Chance and co-workers for animal mitochondria (7, 20).

2 Room temperature difference spectra of cytochrome b-566 in mitochondria from animals (20), higher plants (11), yeast (22), and Neurospora (7) show a minor a-peak at 558 nm, which is usually about one-half of the height of the main peak at 566 nm. Whether the 558 nm peak belongs to b-566 or whether it is a separate low potential component is uncertain and will remain so until b-566 has been highly purified. In the present manuscript, for ease of presentation, we have adopted the nomenclature b-566 (b-558) to refer to this situation.

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Neurospora mitochondria contain additional b-like components reduced only by dithionite. The present paper suggests that the latter is in fact the case and provides spectrophotometric and potentiometric evidence for the presence of a “dithionite-reducible” cytochrome b in both the wild type and poky strains of N. crassa.

**MATERIALS AND METHODS**

**Strains of Neurospora**—The wild type strain, RL21a, was derived from the original Lindegren wild types L1A and L25a (8). The poky strains, NSX f+ a and NS8 f- A were obtained from Dr. D. Luck, The Rockefeller University. f+ is a nuclear gene suppressor of the poky mutant, increasing the growth rate of strains which carry it without restoring the wild type respiratory system (5, 9).

**Growth of Cells**—Cells were grown in aerated liquid cultures at 25°C in Vogel’s minimal medium (10) plus 2% sucrose and were harvested during late exponential phase: 16.5 hours for wild type, 20 to 21 hours for poky f+, and 23 to 24 hours for poky f-.

**Preparation of Mitochondria**—Mitochondria were prepared by osmotic lysis of small enzyme-treated cells followed by two cycles of differential centrifugation (6). The mitochondrial preparations were virtually free of microsomal contamination as judged by the recovery of more than 95% of the mitochondrial protein in a single band following isopycnic sedimentation on discontinuous sucrose gradients of the type used by Dounce et al. for the purification of higher plant mitochondria (12). Mitochondrial protein was determined by the method of Lowry et al. (13) using crystalline bovine serum albumin (Miles Laboratories) as a standard.

**Spectrophotometric Measurements**—Spectra of cytochromes at room temperature and at 77 K were obtained with a scanning split beam spectrophotometer (14). The time course and extent of oxidation and reduction of the cytochromes were measured with a dual wavelength spectrophotometer (14). For purposes of computation in the spectrophotometric experiments, the cytochromes were assumed to be fully oxidized in aerobic mitochondria lacking substrate. The extinction coefficient of cytochrome b was assumed to be 20 mm\(^{-1}\) cm\(^{-1}\) (15).

**Potentiometric Titrations**—Potentiometric titrations were carried out by the method of Dutton et al. (16) making use of a dual wavelength spectrophotometer in combination with an appropriate electrode system.

**RESULTS**

**Spectrophotometric Resolution of Dithionite-reducible Cytochrome b**—The idea that Neurospora mitochondria contain a b-like component reduced only by dithionite was originally based on the observation that total cytochrome b was only about 50% reduced upon anaerobic plus succinate or NADH (4). Proceeding from this result, it is necessary to distinguish between dithionite-reducible b and b-566 (b-558), since the latter cytochrome has a low midpoint potential and is also known to be incompletely reduced by physiological substrates (4, 7, 17).

The approach adopted was to force reduction of b-566 (b-558) by taking advantage of the large increase in its reducibility which can be induced by antimycin plus oxidant (4, 7, 17, 18). The mechanism of the antimycin effect is unknown, but the dithionite-reducible b does remain oxidized under these conditions so that the effect can be used to resolve the two components. Fig. 1 shows spectra for wild type and poky f+ obtained with this approach by using the dual wavelength spectrophotometer and plotting the difference between the oxidation-reduction level in the presence of dithionite and that in the presence of succinate plus antimycin at different measuring wavelengths (with reference to 540 nm). A and B, spectra for wild type and poky f+ mitochondria, respectively. C, standard double beam trace which was repeated at each wavelength pair. Concentrations were: ATP, 0.17 mM; succinate, 10 mM; and antimycin, 1.5 and 0.49 μg per mg of protein for wild type and poky f+, respectively. Dithionite was added as a few grains of the powder. M₉ addition of mitochondria. Mitochondrial protein concentrations were 1.4 and 3.4 mg per ml in the wild type and poky f+ spectra, respectively; 1-cm light path, room temperature.

**Fig. 1.** Spectra of dithionite-reducible cytochrome b obtained with a dual wavelength spectrophotometer by plotting the difference between the oxidation-reduction level in the presence of dithionite and that in the presence of succinate plus antimycin at different measuring wavelengths (with reference to 540 nm). A and B, spectra for wild type and poky f+ mitochondria, respectively. C, standard double beam trace which was repeated at each wavelength pair. Concentrations were: ATP, 0.17 mM; succinate, 10 mM; and antimycin, 1.5 and 0.49 μg per mg of protein for wild type and poky f+, respectively. D, spectra for wild type and poky f+ obtained with this approach by using the dual wavelength spectrophotometer and plotting the difference between the oxidation-reduction level in the presence of antimycin and that in the presence of succinate plus antimycin as a function of measuring wavelength. In the α region, the spectra show a single peak, and in the β region, the spectra show a single peak, at 558 and 559 nm, equivalent to 0.2 nmole of cytochrome b per mg of protein in wild type and about one-fourth that amount (0.05 nmole of cytochrome b per mg of protein) in poky f+.

For both strains, the dithionite-reducible component contributes at least 30 to 50% of the total b-cytochrome absorbance at wavelengths between 558 and 560 nm and the lower absolute concentration in poky merely reflects the deficiency of total cytochrome b in this strain. Fig. 2 shows analogous spectra recorded at 77 K using the split beam spectrophotometer. The spectral peaks, shifted a few nanometers to the blue at low temperature, remain characteristic of a b type cytochrome (α-peaks at 556 nm, β-peaks 527 to 535 nm, and Soret peak at 424 nm). In addition, with the increased resolution at low temperature (19), distinct shoulders are seen associated with the α-peaks in both strains (551 and 562 nm for wild type and 552 nm for poky f+). It is not known whether these shoulders are part of the dithionite-reducible b or whether they are due to a small amount of b-566 (b-558) that remains oxidized in the reference cuvette. The peaks at 543 to 547 nm are presumably due to interference from cytochrome c.

**Potentiometric Titrations**—The spectrum of the dithionite-reducible cytochrome b is similar to that of b-561. Indeed, thus far the main distinction is that the latter is assumed to be completely reduced upon anaerobiosis in the presence of succinate, whereas the former remains oxidized under these conditions.
The sample in the measuring cuvette was reduced with a few grains of dithionite for wild type and poky f+ mitochondria. The reference sample was treated with 10 nm succinate (plus 0.1 mM N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride in the case of wild type) and, after anaerobiosis, antimycin was added at a concentration of 10 µg per ml. At the same time, antimycin was added to the measuring cuvette to eliminate the possibility of interference from anti-mycin-induced spectral shifts. The reference sample was then vigorously pipetted to induce a transient aerobic state, ensuring that the sample had returned to anaerobiosis. The mitochondrial protein concentrations were 4.6 and 7.1 mg per ml in Spectra A and B; 2-hydroxy-1,4-naphthoquinone (17) was used as an aerobic reoxidant for wild type and poky f+ mitochondria. FIG. 3 shows representative potentiometric titrations at 559-540 nm for wild type and poky f+ mitochondria at wavelength pair 559-540 nm.

Although the assumed behavior of b-561 is characteristic of this component in all other types of mitochondria (11, 20-22), strictly speaking, the spectrophotometric data do leave open the possibility that *Neurospora* b-561 is only partially reduced by succinate (plus anaerobiosis). In that case, what we have designated dithionite-reducible cytochrome b would simply be the oxidized portion of b-561 rather than a separate component. In order to eliminate this uncertainty and also to obtain a better estimate of the relative concentrations of the different cytochromes, we carried out potentiometric titrations on *Neurospora* mitochondria with the technique of Dutton et al. (16). This technique has had wide application in resolving cytochromes which have overlapping spectra but which differ in midpoint potential (11, 16, 22-24). Fig. 3 shows representative titrations for wild type and poky f+ mitochondria at wavelength pair 559-540 nm. Indeed the data for both strains do fall on a curve showing three major components indicative of at least three b-cytochromes. The plots are resolved to the right in Fig. 3, permitting determination of *E*m values and relative contributions of the different components. Note that the resolved data show good fit to straight lines of slope close to that for 1-electron oxidation-reduction reactions (i.e. 60 mV per decade change in concentration ratio; see Refs. 11, 16, and 23) as expected for cytochromes. Table I summarizes the results of several experiments as well as the assignment of the measured *E*m values to individual cytochromes (b-561, *E*m = 80 to 90 mV; b-566 (b-558), *E*m = -50 to -70 mV; dithionite-reducible b, *E*m = -140 to -170 mV). Assignment of the highest measured *E*m to b-561 is confirmed by the fact that b-561 is the only b-cytochrome reduced by mild reductants like N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride plus ascorbate (7). Similarly, from the spectrophotometric studies (see above and Refs. 4 and 7), it is known that dithionite-reducible b must be the

![Fig. 2. Low temperature difference spectra showing the dithionite-reducible cytochrome b in wild type and poky f+ mitochondria. The sample in the measuring cuvette was reduced with a few grains of dithionite. The reference sample was treated with 10 mm succinate (plus 0.1 mM N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride in the case of wild type) and, after anaerobiosis, antimycin was added at a concentration of 10 µg per ml. At the same time, antimycin was added to the measuring cuvette to eliminate the possibility of interference from antimycin-induced spectral shifts. The reference sample was then vigorously pipetted to induce a transient aerobic state, ensuring that the sample had returned to anaerobiosis. The mitochondrial protein concentrations were 4.6 and 7.1 mg per ml in Spectra A and B; 2-hydroxy-1,4-naphthoquinone (17) was used as an aerobic reoxidant for wild type and poky f+ mitochondria.](https://example.com/fig2)

![Fig. 3. Representative potentiometric titrations at 559-540 nm for wild type and poky f+ mitochondria. Plots to the left show complete titration data, whereas those to the right show resolution of components using the arithmetic method of Dutton et al. (16). Details of the experimental procedure are given in Refs. 11, 16, and 23. The mitochondria were suspended in standard reaction medium (pH 7.2; see "Materials and Methods") and uncoupled by 2-min soak in ATP (0.17 mM) followed by addition of 1799 (3.3 µM). Titrations were carried out reductively (i.e. by slowly lowering the oxidation-reduction potential). The following oxidation-reduction mediators were present: dianisodurene (42 µM), phenazine methosulfate (42 µM), phenazine ethosulfate (42 µM), pyocyanine (5 µM), duroquinone (67 µM), and 2-hydroxy-1,4-naphthoquinone (17 µM). Reversibility of the titrations was established by control experiments in which it was found that reoxidation by ferricyanide after completion of a titration gave points lying close to the same titration curve. Other control experiments showed that interference due to absorbance changes of the oxidation-reduction mediators was negligible under the present experimental conditions. Mitochondrial protein concentrations were 2.0 and 4.5 mg per ml for wild type and poky f+, respectively. Components (1), (2), and (3) in the figure are considered to be b-561, b-566 (b-558), and the dithionite-reducible cytochrome b, respectively.

![Fig. 3. Representative potentiometric titrations at 559-540 nm for wild type and poky f+ mitochondria. Plots to the left show complete titration data, whereas those to the right show resolution of components using the arithmetic method of Dutton et al. (16). Details of the experimental procedure are given in Refs. 11, 16, and 23. The mitochondria were suspended in standard reaction medium (pH 7.2; see "Materials and Methods") and uncoupled by 2-min soak in ATP (0.17 mM) followed by addition of 1799 (3.3 µM). Titrations were carried out reductively (i.e. by slowly lowering the oxidation-reduction potential). The following oxidation-reduction mediators were present: dianisodurene (42 µM), phenazine methosulfate (42 µM), phenazine ethosulfate (42 µM), pyocyanine (5 µM), duroquinone (67 µM), and 2-hydroxy-1,4-naphthoquinone (17 µM). Reversibility of the titrations was established by control experiments in which it was found that reoxidation by ferricyanide after completion of a titration gave points lying close to the same titration curve. Other control experiments showed that interference due to absorbance changes of the oxidation-reduction mediators was negligible under the present experimental conditions. Mitochondrial protein concentrations were 2.0 and 4.5 mg per ml for wild type and poky f+, respectively. Components (1), (2), and (3) in the figure are considered to be b-561, b-566 (b-558), and the dithionite-reducible cytochrome b, respectively.](https://example.com/fig3)

**Table I**

Midpoint potentials and relative contributions of b-cytochromes in *Neurospora* mitochondria as measured in potentiometric titrations

<table>
<thead>
<tr>
<th>E_m values</th>
<th>Wild type</th>
<th>poky f+</th>
</tr>
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<tbody>
<tr>
<td>b-561</td>
<td>78 ± 2 (61%)</td>
<td>58 ± 3 (33%)</td>
</tr>
<tr>
<td>b-566 (b-558)</td>
<td>-73 ± 8 (19%)</td>
<td>-50 ± 12 (12%)</td>
</tr>
<tr>
<td>Dithionite-reducible cytochrome b</td>
<td>-166 ± 4 (30%)</td>
<td>-144 ± 3 (55%)</td>
</tr>
</tbody>
</table>

The abbreviation used is: E_m, midpoint potential at pH 7.2.
predominant low potential component at the wavelength pair used in the titrations, and also that there should be a smaller contribution from b-566 (b-558). For all three cytochromes the $E_m$ values are in close agreement with those of the analogous b components in animal, yeast, and higher plant mitochondria (11, 16, 22, 23).

An outstanding feature of the potentiometric titrations is that they permit accurate determination of the relative contributions of the different b components. Significantly, the data in Table I show that the proportion of dithionite-reducible b is about twice as great in poky + as in wild type mitochondria (35 and 30%, respectively, at the wavelength pair used) while, on the other hand, the ratio of b-561 to b-556 (b-558) is about the same in the two types of mitochondria. In subsequent experiments, results similar to those for poky + were obtained for poky - with the dithionite-reducible b contributing about 60% of the total b absorbance and with no change in the ratio of b-561 to b-566 (b-558).

**DISCUSSION**

The data presented in this report show that mitochondria from both the wild type and poky strains of N. crassa contain at least three b cytochromes: b-561, b-556 (b-558), and a dithionite-reducible b. b-561 and b-566 (b-558) are apparently analogous to components found in animal and plant mitochondria (11, 20-22) and sometimes referred to as $b_K$ and $b_L$, respectively (20). Some properties of these components in Neurospora mitochondria have been discussed previously (4, 7). A dithionite-reducible component has also been reported for some other types of mitochondria, including beef heart (16, 25), yeast (22), and higher plants (11), and in each case, the measured $E_m$ has been found to be about -100 mv (11, 16, 22). For Neurospora and higher plants, there is good evidence that the dithionite-reducible b is truly a mitochondrial component (and not a microsomal contaminant) since it is present in very pure mitochondrial preparations. The Neurospora mitochondria used in the present work are judged to be free of microsomal contamination by isopycnic sedimentation (see "Materials and Methods"), and it should be added that the gradient-purified mitochondria were tested and found to be identical to those ordinarily used with respect to both the spectrum and concentration of dithionite-reducible b.

The function, if any, of the dithionite-reducible b is not known. The component is considered to be a b-cytochrome on the basis of its characteristic b-cytochrome absorption spectrum (Fig. 2) and its ability to function as a 1 electron carrier in the potentiometric titrations (Fig. 3). However, the term "cytochrome" also implies that the component function physiologically by shuttling between the Fe$^{3+}$ and Fe$^{2+}$ states and this has not been demonstrated in any system. It should be noted that some types of animal mitochondria (e.g., pigeon heart mitochondria) apparently do not have a dithionite-reducible b (16, 20), a fact which indicates that the component cannot be an essential part of the respiratory chain. Indeed, the relatively high concentration of the dithionite-reducible b in poky, which is known to accumulate precursor and nonfunctional forms of other respiratory chain components (26, 27), lends support to the view that it is nascent or damaged cytochrome b. In this regard, it is interesting to note the recent observation of Sato et al. (28) that a component with properties similar to dithionite-reducible b increases in concentration at the expense of the respiratory chain b-cytochromes in L cells treated with ethidium bromide (a substance which blocks formation of functional electron transport systems).

As expected, the concentration of all three b-cytochromes is lower in poky than in wild type mitochondria. However, it can be concluded (a) that the ratio of b-561 and b-556 (b-558) remains 1:1 in both strains (from spectra of antimycin-treated mitochondria reduced with succinate (4), with support from the potentiometric titrations reported here), and (b) that the dithionite-reducible b is present in more variable concentrations, comprising a greater proportion of the total b in poky than in wild type. The presence of a separate dithionite-reducible component, although suggested previously (4), was overlooked by von Jagow et al. (7) and this oversight probably accounts for the unusually high ratio of b-561 to b-566 (b-558) which they reported for poky mitochondria. Indeed, it should be added that, apart from interpretation, the data in the von Jagow study agree very well with the concept of a dithionite-reducible component since their spectra also indicate incomplete reduction of cytochrome b by succinate. It is possible, however, that their wild type mitochondria contained somewhat less, and their poky mitochondria somewhat more, dithionite-reducible b than those used in the present work.

The measured $E_m$ values of the three b species are summarized in Table I. As noted in an earlier study, the $E_m$ values measured by the techniques now available may be less accurate than the precision of the measurements suggests (11). However, the data are sufficient to show that there is no gross difference in the $E_m$ values between the wild type and poky + strains. This result is in agreement with the earlier generalization that the same b components are present in both strains and that, except with respect to concentration, they are not significantly altered by the poky mutation (4).

The sequence of electron transport reactions in the b region of Neurospora is an important problem which must now be approached by kinetic experiments. The contention of von Jagow et al. (7) that the sequence is b-561 to b-566 (b-558) with the latter cytochrome located directly on the substrate side of "site II" is premature. Moreover, this contention was based primarily on experiments with animal mitochondria purporting to show "heme-heme interaction" between b-566 (b-558) and cytochrome c, and participation of b-556 (b-558) in energy transfer at site II (29-31). Virtually all of this evidence has since been brought into question (32-37, 11, 17). At present, the only evidence bearing on the ordering question in Neurospora mitochondria is in fact the potentiometric data in this report and these data suggest that b-561 (the high potential species) is on the oxygen side of b-566 (b-558).

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