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SPECTRAL STUDY OF *b* CYTOCHROMES IN YEAST MITOCHONDRIA AND INTACT CELLS

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SUMMARY

Three types of *b* cytochromes are demonstrated in *Candida utilis* mitochondria. One of these *b* cytochromes has a symmetrical α-band at 561.5 nm at room temperature. This *b* cytochrome is readily reduced either by anaerobiosis or by cyanide treatment in the presence of glycerol 1-phosphate or succinate both in coupled and uncoupled mitochondria. The second *b* cytochrome has a double α-band at 565 nm and 558 nm. This *b* cytochrome is readily reduced either by anaerobiosis or by cyanide treatment in the presence of glycerol 1-phosphate or succinate in coupled mitochondria, but in uncoupled mitochondria it is slowly reduced after anaerobiosis and this reduction rate is enhanced by antimycin A addition. Thus the oxidation–reduction state of this cytochrome is energy dependent. The first cytochrome is spectroscopically identified as cytochrome b_K and the second as cytochrome b_T. The third *b* cytochrome has an α-band around 563 nm (b_{563}) and is reduced slowly after anaerobiosis in uncoupled mitochondria but faster than the b_T. Further properties of this component are not known. Midpoint potentials of cytochromes b_T, b_{563} and b_K are approximately -50 mV, $+5$ mV, and $+65$ mV, respectively.

In intact cells, cytochrome b_T is reduced immediately after anaerobiosis or cyanide treatment, and rapidly oxidized when uncoupler is added. Addition of antimycin A instead of uncoupler to the anaerobic cells causes oxidation of mainly cytochrome b_T while addition of antimycin A to the aerobic cells results in a reduction of the cytochrome b_T.

INTRODUCTION

Considerable effort has been extended in searching for the role of cytochrome *b* in energy transduction in the respiratory chain by using intact mitochondria[1–5], or submitochondrial particles[6–8]. The existence of two chemically distinct species of *b* cytochromes has been demonstrated by poising the system with oxidation–reduction dyes in rat liver[5] and pigeon heart mitochondria[2] and of three chemically distinct

Abbreviations: FCCP, trifluoromethyl oxycarbonylphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzinidazole.

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species in beef heart[9] and pigeon heart[8,10] submitochondrial particles. Wilson and Dutton[5,10] observed that one of these two *b* cytochromes (b_T) has an energy-dependent oxidation–reduction midpoint potential and postulated that it directly participates in transduction of the electrochemical potential of electron transport to chemical potential suitable for ATP synthesis. Recently, Sato *et al.*[11–13] distinguished spectroscopically two *b* cytochromes in pigeon heart mitochondria. One of these *b* cytochromes has a single symmetric α-band at 561 nm at room temperature (at 77 °K, it has an α maximum at 558 nm), and the other one has a double α-band at 565 nm and 558 nm (at 77 °K, it has a double α-band at 562.5 nm and 555 nm). Potentiometric titration identified the former as cytochrome b_K and the latter as b_T[13].

It has also been shown that plant mitochondria have three types of *b* cytochromes (b_{562}, b_{557}, and b_{553})[14], and one of these *b* cytochromes was suggested to be involved in energy conservation[15]. None of these, however, has been shown to have an energy-dependent midpoint potential change[16]. In view of the possibility of a direct involvement of the *b* cytochromes (particularly of b_T) in energy conservation in animal mitochondria it is of considerable interest to determine the behavior of these cytochromes in yeast cells and mitochondria.

In this communication we report the existence of three spectrally and functionally different forms of *b* cytochromes in mitochondria isolated from *Candida utilis* cells. One of these cytochromes has a double α-band at 565 and 558 nm, and is spectrally and functionally quite similar to that of cytochrome b_T observed in pigeon heart mitochondria[11–13]. The double α-band of this *b* cytochrome is particularly interesting because the role of this hemoprotein in energy conservation may depend upon the special environment of the heme.

METHODS AND MATERIALS

Yeast cells and mitochondria: *C. utilis* cells were grown aerobically for 24 h at 30 °C in a synthetic culture medium[17] with 1.5 % ethanol as a carbon source. Mitochondria were prepared by a combined enzymatic[18,19] and mechanical procedure[20], and suspended in 0.6 M mannitol, 0.1 mM EDTA, and 50 mM morpholinopropane sulfonate (pH 7.0).

Analytical methods: Difference spectra of cytochromes were recorded at room temperature and at the temperature of liquid nitrogen in the two-chambered cuvette of a Johnson Foundation double-beam (split beam) spectrophotometer[21]. Measurements of the steady state absorbance change of cytochromes were made with an Aminco–Chance dual wavelength spectrophotometer and a Hitachi Perkin–Elmer 356 two wavelength spectrophotometer.

Potentiometric measurements of cytochromes were performed as described by Wilson and Dutton[7]. The oxidation–reduction mediators were those used by Wilson and Dutton, namely 16 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), 17 μM diaminodurene, 17 μM phenazine ethosulfate, 17 μM phenazine methosulphate (K and K Laboratories, Plainview, N.Y.), 5 μM pyocyanine, 30 μM duroquinone (Eastman Organic Chemicals, Rochester, N.Y.), 17 μM 2-hydroxy-1,4-naphthoquinone.

Chemicals: 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) was a gift from Dr T. Galleotti. Antimycin A was obtained from Sigma Chemical Co. and dissolved in dimethyl formamide.

Protein was determined according to Kröger and Klingenberg[22] with bovine serum albumin as standard.

RESULTS

The spectral properties of b cytochromes in C. utilis mitochondria

As shown in Fig. 1, when a suspension of *C. utilis* mitochondria is supplemented with glycerol 1-phosphate in the presence of piericidin A, respiration is initiated and the *b* cytochromes become partially reduced as measured at 563 nm *minus* 575 nm. An aerobic to anaerobic transition gives an increased reduction which shows rapid (50 %) and slow (25 %) phases up to 20 min after anaerobiosis. Addition of antimycin A causes almost no spectral change at this wavelength pair. Further addition of dithionite completely reduces the *b* cytochromes. Identical experiments were carried out with the various measuring wavelengths with a fixed reference wavelength at 575 nm. The difference in absorbance between the points designated A through H (Fig. 1) were plotted as a function of measuring wavelength (Figs 2-I and 2-II). The spectrum of the *b* cytochrome reduced immediately after anaerobiosis (B−A) has an α absorption band at 561.5 nm, while the *b* cytochrome(s) reduced slowly after the anaerobiosis (F−B) has a complex α-band with a maximum at 563 nm and shoulders

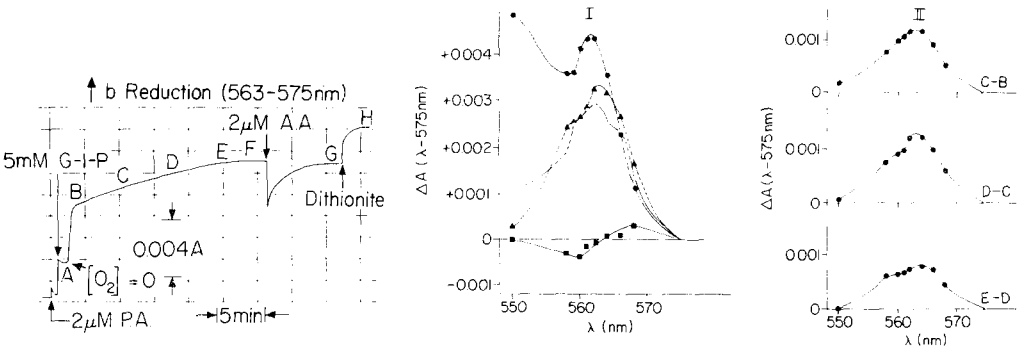

Fig. 1. The effect of substrate and antimycin A on the degree of reduction of *b* cytochromes (as measured at 563–575 nm) in *C. utilis* mitochondria. The mitochondria were suspended at 1.6 mg protein per ml in a medium containing 0.225 M mannitol, 0.075 M sucrose, 0.2 mM EDTA and 25 mM morpholinopropane sulfonate, pH 7.2. The reagents were added at the indicated times and final concentrations. P. A., piericidin A; G-1-P, glycerol 1-phosphate; A.A., antimycin A.

Fig. 2. The spectral properties of the *b* cytochromes in *C. utilis* mitochondria. The mitochondria were suspended and treated as described in the legend of Fig. 1. A series of experiments were carried out in which the measuring wavelength was varied but the reference wavelength was left at 575 nm. (I) The differences in absorbance between the specified spectra are obtained directly by plotting the absorbance changes designated A through H in Fig. 1. Therefore, ●—● (B−A) represents the spectrum of the *b* cytochrome which is reduced by anaerobiosis in the presence of glycerol 1-phosphate; ▲—▲ (F−B) represents the spectrum of *b* cytochromes which are reduced in 18 min after anaerobiosis; ■—■ (G−F), the spectral shift induced in the *b* cytochrome spectrum by the addition of antimycin A to 18-min anaerobic mitochondria; ○—○ (H−G), the *b* cytochrome reduced when dithionite is added to anaerobic mitochondria in the presence of glycerol 1-phosphate and antimycin A. (II) Curve C−B represents the spectrum of *b* cytochrome which is reduced in 5 min after anaerobiosis in the presence of glycerol 1-phosphate; Curve D−C, the spectrum of *b* cytochrome reduced between 5 and 10 min after anaerobiosis in the presence of glycerol 1-phosphate; Curve E−D, the spectrum of *b* cytochrome reduced between 10 and 15 min after anaerobiosis in the presence of glycerol 1-phosphate.

at 565 nm and 558 nm. When the differences in absorption between the points C and B, D and C, and E and D (Fig. 2-II) were plotted as a function of measuring wavelength, the spectrum of the *b* cytochrome reduced in 5 min after anaerobiosis (C−B) has an almost symmetric α-band with a maximum around 563 nm; the *b* cytochrome reduced between 10 min and 15 min after anaerobiosis (E−D) shows a spectrum with two α maxima at 565 and 558 nm which is quite similar to that of cytochrome b_T observed in pigeon heart mitochondria[11-13]. The spectrum of *b* cytochromes reduced between 5 min and 10 min after anaerobiosis (D−C) is superimposed upon those of the two *b* cytochromes as mentioned above. The relative ease of reducibility of the *b* cytochromes by substrates shown in Figs 1 and 2 suggest a different midpoint potential, with potentials becoming more negative in the order of the *b* cytochrome having a peak at 561.5 nm, the *b* cytochrome having a peak around 563 nm and the *b* cytochrome having a double peak at 565 and 558 nm.

The addition of a saturating amount of antimycin A (1.5 nmoles/mg protein) to the mitochondrial suspension causes a shift in the absorption maximum of *b* cytochromes as shown in Fig. 2-I (G−F) which is very similar to that reported by Pumphrey[23] in electron transfer particles (ETP) and Sato *et al.*[13] in pigeon heart mitochondria. The point of zero absorbance change approaches the maximum of absorption band which shifted and, thus, antimycin A-induced absorbance shift seems to arise from the absorption band with a maximum near 562 nm.

Addition of dithionite after treatment with antimycin A gives further reduction of *b* cytochromes (H−G) which shows a complex spectrum with a peak at 563 nm and shoulders at 565 and 560 nm. More than two types of *b* cytochromes may be involved in this spectrum. Cytochrome *c* is also reduced by dithionite as evidenced by the increase in absorbance at 550 nm.

The energy-dependent reduction of b cytochromes in cyanide-treated mitochondria

The mitochondrial suspension was treated with piericidin A, ascorbate, TMPD and dicumarol, and then 30 s later with KCN to reduce cytochromes *a*, *c* and c_1. This eliminates possible contribution of these cytochromes in the *b* cytochrome region. As shown in Fig. 3A, the addition of glycerol 1-phosphate causes a partial reduction of the *b* cytochrome (40 %) as measured at 563 nm *minus* 575 nm. This reduction is increased to 70 % when antimycin A is added. Finally dithionite reduces completely the cytochromes of the *b* type.

When the suspension was treated with piericidin A, ascorbate, TMPD and ADP in the presence of phosphate to permit endogenous ATP formation and then 30 s later KCN was added, the *b* cytochrome is 30 % reduced without added substrate (Fig. 3B). Subsequent addition of glycerol 1-phosphate gives increased reduction of *b* cytochrome to 60 %, which falls to 40 % when uncoupler is added. Thus, a part of the absorbance of the *b* cytochromes, (about 20 %) under these conditions, in cyanide treated yeast mitochondria, changes in a manner consistent with an energy dependent half-reduction potential.

To clarify the spectral properties of *b* cytochromes involved in these reactions, difference spectra were measured at 77 °K by the trapped steady-state method[1] between the points designated B and A, C and B, D and C, E and A and G and F in Fig. 3 (Fig. 4). The portion of *b* cytochrome reduced by glycerol 1-phosphate in uncoupled mitochondria (B–A) has a symmetrical α-band at 558 nm, a β-band at

529 nm and a Soret band at 427 nm, which is thereby identified with cytochrome b_K[11-13]. The *b* cytochrome reduced by antimycin A addition has two α maxima, a sharp one at 562 nm and a rather broad one at 557 nm. This spectrum may be interpreted as the superimposition of the two *b* cytochromes; mainly, the *b* cytochrome with double α maxima (cytochrome b_T), and partly the *b* cytochrome with α maxima

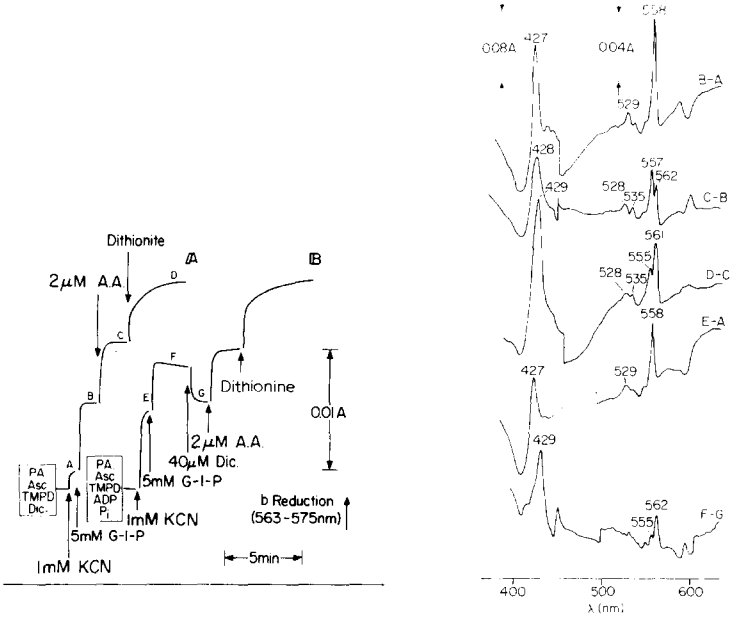

Fig. 3. The effect of substrate on the degree of reduction of the *b* cytochromes in coupled and uncoupled mitochondria isolated from *C. utilis*. The mitochondria were suspended in the assay medium at 2.1 mg protein per ml and supplemented with 3 μM piericidin A, 2 mM sodium ascorbate and 60 μM TMPD. To the suspension (I), 40 μM dicumarol was then added, and to the suspension (II) 1 mM ADP and 2 mM phosphate were added to permit ATP formation. The reagents were added at the indicated times and final concentrations. P.A., piericidin A; Asc, ascorbate; Dic., dicumarol; G-1-P, glycerol 1-phosphate; A.A., antimycin A.

Fig. 4. The absorption spectra of *b* cytochromes in *C. utilis* mitochondria at 77 °K. The *C. utilis* mitochondria were suspended at 7.1 mg protein per ml and treated as given in the legend of Fig. 3. Spectrum B—A: the reference sample was withdrawn at the designated point A in Fig. 3, where the sample was treated with piericidin A, ascorbate, TMPD, dicumarol and KCN, and injected into the spectrophotometer cuvette which has been pre-cooled to liquid nitrogen temperature. The measure sample was similarly treated but was withdrawn 1 min after the addition of 5 mM glycerol 1-phosphate (point B in Fig. 3). Therefore this shows the spectrum of *b* cytochrome reduced when glycerol 1-phosphate was added. Spectrum C—B: the reference material was the same as the measure sample in Spectrum B—A (withdrawn at the point B in Fig. 3). The measure sample was withdrawn 1 min after the addition of antimycin A in the presence of glycerol 1-phosphate (point C in Fig. 3) and similarly treated as described in the legend of Spectrum B—A. Spectrum D—C: the reference material was the same as the measure sample in Spectrum C—B. The measure sample was withdrawn 4 min after the addition of dithionite in the presence of glycerol 1-phosphate and antimycin A (point D in Fig. 3). Spectrum E—A: the reference sample was withdrawn at the point A in Fig. 3 and similarly treated as the reference sample in Spectrum B—A. The measure sample was withdrawn at the point E in Fig. 3 where the suspension was treated with 3 μM piericidin A, 2 mM ascorbate, 60 μM TMPD, 1 mM ADP, 2 mM phosphate and 30 seconds later 1 mM KCN. Spectrum F—G: the reference sample was withdrawn at the point G in Fig. 3 where 40 μM dicumarol was added in the presence of glycerol 1-phosphate. The measure sample was withdrawn before the addition of dicumarol (point F in Fig. 3).

at 561.5 nm or at 563 nm at room temperature. The *b* cytochrome reduced by dithionite addition in this preparation shows two maxima; one is broad at 561 nm and the other is small at 555 nm. This spectrum may involve two *b* cytochromes; one is cytochrome b_T and the other is cytochrome *b* with α maximum around 563 nm at room temperature.

The *b* cytochrome reduced by cyanide treatment of mitochondria having oxidized ascorbate *plus* TMPD in the presence of ADP and P_i (E–A) shows the symmetric α-band at 558 nm, which is identified as cytochrome b_K. It may be reduced by energy-linked reversed electron transfer from phosphorylation Site III (see ref. 24). The *b* cytochrome which had been reduced by glycerol 1-phosphate in coupled mitochondria and oxidized by uncoupler (F–G) has a double α-band at 562 nm and 555 nm, which is identified as cytochrome b_T[11–13]. The difference spectrum between the coupled and uncoupled mitochondria both in the presence of substrate and cyanide shows the essentially same spectrum as in trace F–G.

Effect of uncoupler and antimycin A on the redox state of b cytochrome in intact yeast cells

As shown in Fig. 5, when the suspension of intact cells is supplemented with KCN, rapid reduction of *b* cytochromes is seen. The *b* cytochromes are reduced 85 % at this wavelength pair (430 nm *minus* 412 nm). The addition of uncoupler, TTFB, causes rapid oxidation of a part of *b* cytochromes (25 %). Cytochromes *a* and $c + c_1$ remained unaffected. However, the cytochromes a_3–CN complex measured at 592 *minus* 630 nm is gradually formed after cyanide treatment and this formation is enhanced by uncoupler addition.

Antimycin A addition seems to reduce completely the *b* cytochromes in aerobic

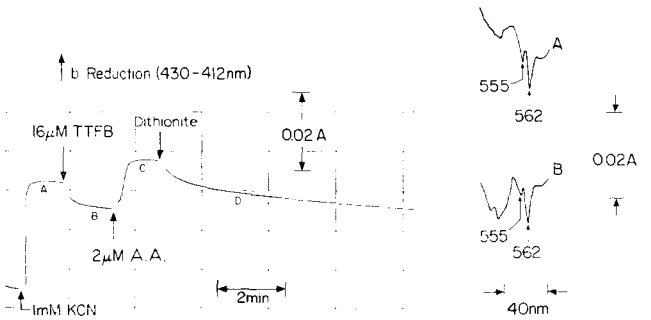

Fig. 5. The effect of the addition of uncoupler and antimycin A (A.A.) on the degree of reduction of the *b* cytochromes (as measured at 430 nm *minus* 412 nm) of *C. utilis* cells. Cells were suspended in the oxygen saturated 50 mM phosphate buffer, pH 6.8, at the concentration of 3.5 mg dry weight per ml. The reagents were added at the indicated times and final concentrations.

Fig. 6. The absorption spectra of *b* cytochromes oxidized by the addition of uncoupler (A) and antimycin A (B), in *C. utilis* cells at 77 °K. The *C. utilis* cells were suspended at 20 mg dry weight per ml in the oxygen bubbled 50 mM phosphate buffer, pH 6.8. Spectrum A: the reference sample was withdrawn 30 s after the addition of KCN (point A in Fig. 5) and injected into a spectrophotometer cuvette which had been pre-cooled to liquid nitrogen temperature. The measure sample was similarly treated but was withdrawn 1 min after the addition of TTFB in the presence of KCN (point B in Fig. 5). Spectrum B: the reference sample was withdrawn 1 min after the addition of antimycin A in the presence of KCN and TTFB, and treated similarly as described in the legend of Spectrum A. The measure sample was similarly treated but was withdrawn about 3 min after the addition of dithionite in the presence of KCN, TTFB and antimycin A.

state, after which removal of oxygen by dithionite addition causes partial oxidation (35 %) of *b* cytochromes as first observed in yeast cells by Chance[25] in 1952 and later by Kovac *et al.*[26].

To clarify the property of the *b* cytochrome oxidized by uncoupler and reduced by antimycin A, the sample was taken at the indicated points A through D in Fig. 5 and the difference spectra were measured at 77 °K[1]. As shown in Fig. 6A, the *b* cytochrome oxidized by addition of uncoupler (A – B in Fig. 5) has a double α-band at 562 and 555 nm. The *b* cytochrome oxidized by aerobic to anaerobic transition in the presence of antimycin A as shown in Fig. 6B (D – C in Fig. 5) has also a double α-band at 562 and 555 nm. In this spectrum no spectral shift of the *b* cytochrome due to antimycin A is involved since both measure and reference samples contain the antibiotic. The difference spectrum between the presence and absence of KCN in the aerobic, TTFB-treated yeast cells at 77 °K (not shown) shows a single α-peak of *b* cytochrome at 558 nm, indicating the presence of cytochrome b_K in these cells.

Potentiometric titration of reduction of b cytochromes

Potentiometric titration of reduction of *b* cytochromes can be carried out as described by Wilson and Dutton[5]. Fig. 7 describes the oxidation–reduction potentials of *b* cytochromes in *C. utilis* mitochondria. The complex curve describes the course of reduction of the *b* cytochromes at 562 nm *minus* 575 nm. Accurate midpoint values cannot be assigned to these components since the values are too close with each other and there is some spectral interference from other cytochromes. Moreover, the endogenous substrates contained in yeast mitochondria make the oxidation–reduction titration more difficult. However, this curve is very similar to those obtained by Dutton *et al.*[9] and Lindsay *et al.*[8] for submitochondrial particles and suggests the existence of three cytochrome *b* components. Redox titrations carried out with various measuring wavelengths with a fixed reference wavelength at 575 nm (Table I)

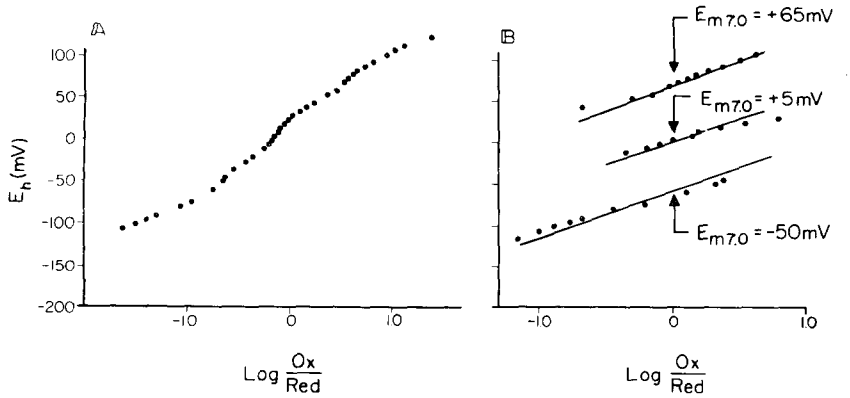

Fig. 7. The course of reduction of *b* cytochromes at 562 nm and 575 nm in *C. utilis* mitochondria as a function of oxidation–reduction potential. *C. utilis* mitochondria were suspended at 2.3 mg protein per ml in 0.225 M mannitol, 0.075 M sucrose, 0.2 mM EDTA and 25 mM morpholinopropane sulfonate (pH 7.0); 17 μM phenazine methosulfate, 17 μM phenazine ethosulfate, 30 μM duroquinone. A reductive titration was done by using NADH and sodium dithionite as reductant. The logarithm of the ratio of the oxidized to the reduced is for the total absorbance change from +200 mV to −175 mV.

suggest that the *b* cytochrome with a single α-band at 561.5 nm has the most positive half-reduction potential among these three *b* cytochromes, and the *b* cytochrome with a double α-band has a more negative half-reduction potential as has been shown in pigeon heart and rat liver mitochondria[2,5,13]; the remaining *b* cytochrome has an intermediate redox potential among three. From several titrations at these wavelength pairs, the following values of mid-point potentials ($E_{m\,7.0}$) were obtained; +65 ± 15 mV for cytochrome b_K, +5 ± 15 mV for cytochrome b_{563}, and −50 ± 15 mV for cytochrome b_T. Almost the same $E_{m\,7.0}$ values were obtained for uncoupled mitochondria or mitochondria supplemented with 2.5 mM ATP.

TABLE I

RELATIVE CONTRIBUTION OF THREE COMPONENTS TO VARIOUS WAVELENGTHS

The mitochondria were suspended in 0.225 M mannitol, 0.075 M sucrose, 0.2 mM EDTA and 25 mM morpholinopropane sulfonate, pH 7.0. The α-band was assayed with a measuring wavelength at 561, 563 and 565 nm with a reference wavelength at 575 nm using 17 μM diaminodurol, 17 μM phenazine methosulphate, 17 μM phenazine ethosulphate, 30 μM duroquinone and 17 μM 2-hydroxy-1,4-naphthoquinone as redox mediators (see Fig. 7). Reductive titration was done using NADH (and lower than about 0 mV, sodium dithionite) as reductant.

Potential span (mV)	*Percentage*			$E_{m\,7.0}$ *(mV)*	*Component*
	λ = 561 nm	*λ = 563 nm*	*λ = 565 nm*		
180 → 40	60	50	43	+65 ± 15	b_K
40 → −10	23	27	24	+ 5 ± 15	b_{563}
−10 → −150	17	23	33	−50 ± 15	b_T

DISCUSSION

In intact mitochondria from mammalian or avian sources, the respiratory chain has been shown to contain at least two species of cytochromes *b*[2,5,11–13,27] and they have recently been assigned redox potential[5] and kinetic properties[2]. One of these *b* cytochromes (b_K) has a symmetrical α-band at 561 nm, the other one (b_T) has a double α-band at 565 and 558 nm. In the present investigation it has become clear that *C. utilis* mitochondria also contain these two types of *b* cytochromes as found in beef heart[8] and pigeon heart[9] submitochondrial particles. The properties of the former two *b* cytochromes seem to be essentially the same as observed in pigeon heart mitochondria[11–13]. The *b* cytochrome with a symmetric α-band at 561.5 nm (cytochrome b_K) is readily reduced by glycerol 1-phosphate or succinate in both coupled and uncoupled mitochondria. In intact cells it is readily reduced by the addition of cyanide or by anaerobiosis.

The *b* cytochrome with double α maxima at 565 nm and 558 nm (cytochrome b_T) is readily reduced by these substrates in coupled mitochondria, but in uncoupled mitochondria it is quite slowly reduced in the presence of substrates after anaerobiosis but not by the treatment with cyanide. In intact cells it is readily reduced, but not fully, by cyanide treatment or by anaerobiosis, and gradual oxidation may follow anaerobiosis.

Although data presented in Fig. 3 and Fig. 5 suggest that the midpoint potential of cytochrome b_T in yeast mitochondria is also energy dependent and becomes

more positive when mitochondria are in the energized state, (*cf.* refs 2, 5, 9 and 10) the value of $E_{m\,7.0}$ titrated in the presence of added ATP was the same as that measured in uncoupled mitochondria (*cf.* ref. 16). This could be explained as follows: *C. utilis* mitochondria show extremely low ATPase activity (0–2 nmoles ATP hydrolysed/min per mg protein) in the absence of added magnesium at pH 7.0 (T. Ohnishi, unpublished observation), as reported in *Saccharomyces* mitochondria[19,28]. Furthermore the ATPase activity is not significantly stimulated by the addition of uncouplers such as trifluoromethyl oxycarbonylphenylhydrazone (FCCP) or 1799, although these uncouplers release the state-4 respiration to the level of State 3 respiration or even higher. This is good evidence to show that added ATP is poorly accessible to the uncoupler-stimulated ATPase. Thus in a reaction medium with a high external phosphate potential, the level of endogenous high-energy intermediate ($X \sim I$) remains too low to cause a change in the midpoint potential of cytochrome b_T because of the slow interaction between the exogenous ATP and the respiratory chain in the absence of added Mg^{2+}. A similar situation occurs in the energy-dependent electron reverse from glycerol 1-phosphate to endogenous pyridine nucleotide in *C. utilis* mitochondria. Considerable reduction of endogenous pyridine nucleotide was observed when the energy was supplied from high-energy intermediate produced at Site II or III, while it is not obtained by the addition of ATP in the absence of the respiration (T. Ohnishi, unpublished results). It should be mentioned here that a much higher phosphate potential is needed to shift cytochrome b_T from the low midpoint potential form to the high midpoint potential form (N. Sato, unpublished observation) than to cause an energy-dependent electron reverse (Chance[9], Klingenberg[30]).

The antimycin A effect on the spectral shift of *b* cytochrome is also observed in *C. utilis* mitochondria as in mammalian mitochondria[13,23,31]; the spectrum of reduced *b* cytochrome having an α-maximum around 562 nm, is shifted to longer wavelength but there is no measurable effect on the spectrum of *b* cytochrome having a double α-maximum. The observed spectral shift is very small (7 % of total absorbance change of *b* cytochromes). Antimycin A is reported to prevent the ATP dependent positive shift of the cytochrome b_T midpoint potential[10] and to stabilize a respiration dependent shift of the cytochrome b_T midpoint potential[32,33]. This is consistent with the observation that when antimycin A is added to intact anaerobic yeast cells (no respiration) the cytochrome b_T becomes more oxidized. The oxidation may be attributed to an inhibition of the interaction of the cytochrome b_T with the cellular ATP resulting in its midpoint potential changing to a more negative value. On the other hand when the antimycin A is added to respiring yeast cells or uncoupled mitochondria the cytochrome b_T becomes reduced as a result of the respiration-dependent change to a more positive midpoint potential[32,33].

The third form of *b* cytochrome, which has an α maximum around 563 nm, is slowly reduced after anaerobiosis in uncoupled mitochondria, but is still faster than cytochrome b_T remains to be investigated. In yeast mitochondria a considerable amount of *b* cytochrome is not reduced in the anaerobic condition but is reducible only by dithionite addition (Fig. 2). The role of this dithionite-reducible cytochrome is also unknown but it seems to represent inactive forms of cytochrome *b* generated during isolation of the mitochondria.

It seems of interest that the observed split of the α-band of cytochrome b_T found in pigeon heart mitochondria[12,13,31], in ascites tumor cells[34] and in yeast mitochondria

in this paper is greater than any other previously shown. This split of α-band may depend upon the special environment of the heme related with energy conservation.

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