# TWO *b* CYTOCHROMES OF PIGEON HEART MITOCHONDRIA

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## 1. Introduction

A direct role of cytochrome *b* in mitochondrial electron transport and energy conservation has been postulated by Chance and coworkers [1, 2] and by Slater and coworkers [3]. These postulates have been based on the anomalous kinetic [4, 5] and spectroscopic [2–6] behavior of the *b* cytochrome in submitochondrial particles, in intact mitochondria, and in the presence of cations [see 7]. Recently Wilson and Dutton [8] have used potentiometric measurement of the oxidation–reduction midpoint potentials of the cytochromes to demonstrate the existence of two chemically distinct species of *b* cytochrome in rat liver [8] and pigeon heart [9] mitochondria, one of which has an energy dependent oxidation–reduction midpoint potential. Kinetic evidence has also been obtained by Chance et al. [9, 10] for the existence of two species of *b* cytochromes in pigeon heart mitochondria.

It is the purpose of this communication to present the spectral properties of two *b* cytochromes of pigeon heart mitochondria. One of these cytochromes has a single symmetric alpha band and the other one has a double alpha band. The properties of these two *b* cytochromes may resolve the complex behavior of *b* cytochrome in the mitochondrial respiratory chain, which was observed by Chance and coworkers [2, 6, 7] and Slater and coworkers [11, 12].

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## 2. Materials and methods

Pigeon heart mitochondria were prepared in 0.225 M mannitol–0.075 M sucrose–0.2 mM EDTA–0.05 M tris-Cl, pH 7.4, according to the method of Chance and Hagihara [13]. Measurements of the steady state absorbance change were made with an Aminco-Chance dual wavelength spectrophotometer and a Perkin-Elmer 356 two wavelength spectrophotometer. Difference spectra of cytochromes were recorded at the temperature of liquid nitrogen in the two chambered cuvette of a Johnson Foundation double beam (split beam) spectrophotometer [14]. *N,N,N′,N′*-Tetramethyl paraphenylenediamine (TMPD) and l-ascorbic acid were purchased from Sigma Chem. Co., and were used in aqueous solutions. The ascorbate and ATP solution were neutralized to pH 6.8 with KOH and tris, respectively. Protein was determined by the buiret method with bovine serum albumin as standard [15].

## 3. Results

### 3.1. *The spectral properties of the b cytochromes*

The suspension of pigeon heart mitochondria was treated with KCN, ascorbate and TMPD to reduce the cytochrome $c_1$, *c*, *a* and $a_3$ and to block the ATP responses in this part of the chain [16]. As shown in fig. 1a, the subsequent addition of succinate causes a partial reduction of the *b* cytochromes as measured at 564 nm minus 575 which increases when ATP is added. The spectra of the *b* cytochromes were measured in identical experiments with various measuring

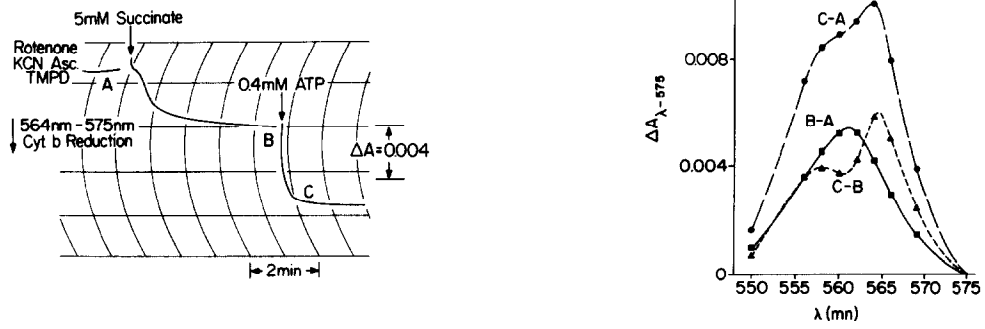


Fig. 1. (a). The effect of substrate and ATP on reduction of *b* cytochromes (564–575 nm) in pigeon heart mitochondria ($PHM_W$). The $PHM_W$ were suspended at 1.8 mg protein/ml in 0.225 M mannitol–0.075 M sucrose–0.2 mM EDTA–0.05 M tris-HCl, pH 7.4 (MSET), and were treated with 2.5 μM rotenone, 5 mM KCN, 5 mM ascorbate and 80 μM TMPD. (b) Spectral properties of *b* cytochromes in pigeon heart mitochondria. The experiment of fig. 1a was carried out such that the measure wavelength was varied but the reference wavelength was fixed at 575 nm. The absorbance changes obtained after addition of succinate in the experimental conditions of fig. 1a and after the addition of ATP are plotted as function of the measuring wavelength. (B–A) *b* cytochrome reduced by succinate; (C–A) *b* cytochromes reduced by succinate and ATP; (C–B) *b* cytochrome reduced when ATP is added to mitochondria in the presence of succinate.

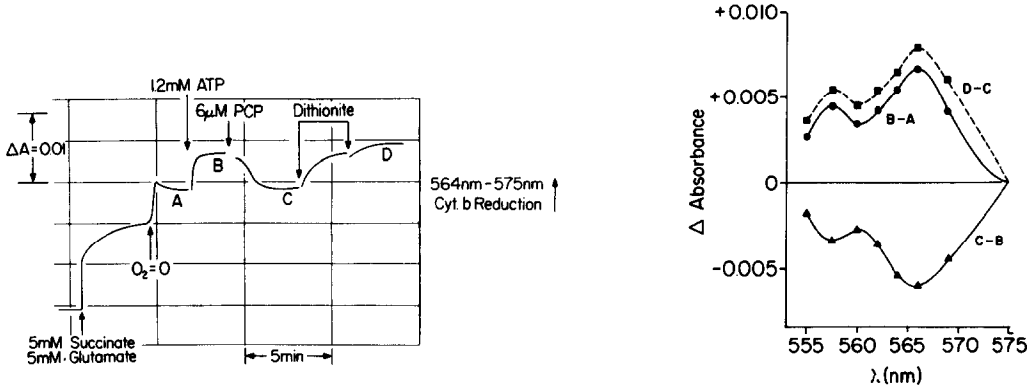


Fig. 2 (a). The effect of energy state on the degree of reduction of *b* cytochromes (564–575 nm) in pigeon heart mitochondria. The $PHM_W$ were suspended at 2.7 mg protein/ml in the MSET. (b) Spectra of the *b* cytochrome whose reduction and oxidation are under the control of energy state of mitochondria. Experimental conditions are the same as in fig. 2a. A series of experiments was carried out at various measuring wavelengths with a fixed reference wavelength at 575 nm. At the time designated by the letters A, B, C and D (fig. 3), the absorbance changes were plotted. (B–A) reduction of *b* cytochrome by 1.2 mM ATP; (C–B) oxidation of *b* cytochrome by 6 μM pentachlorophenol; (D–C) reduction of *b* cytochrome by dithionite.

wavelengths. In fig. 1b the spectra are plotted as the absorbance change between the mitochondria treated with KCN, ascorbate and TMPD (A) and the points designated on fig. 1a as B and C. The *b* cytochrome reduced by succinate addition (B–A) has a maximum at 561 nm and this increases in absorbance and the maximum shifts to 564 nm on addition of ATP (C–A). The *b* cytochrome reduced by ATP addition (C–B)

has an absorption band with two maxima, one at 565 nm and the other at 558 nm, clearly distinguished from cytochrome $c_1$ which was in any case maintained reduced by ascorbate TMPD [15], as verified by fig. 2 a and b.

### 3.2. *The spectral properties of low potential cytochrome b*

As shown in fig. 2a, when the mitochondrial suspension is supplemented with succinate and glutamate, respiration is initiated and the *b* cytochromes become partially reduced (as measured at 564 nm minus 575 nm). When the oxygen in the medium is exhausted the aerobic to anearobic transition gives an increased reduction which displays a slight overshoot and then stabilized with the *b* cytochromes approximately 60 to 75% reduced. Addition of ATP caused a reduction of the cytochromes (to 90%) which is reversed when uncoupler is added. Dithionite addition completely reduces the *b* cytochromes. The spectra of the *b* cytochromes were measured in identical experiments with various measuring wavelengths. The differences in optical density between the points designated A through D (fig. 2a) were then plotted as a function of the measuring wavelength (fig. 2b). The spectrum of the *b* cytochrome reduced by ATP addition (B–A) has two maxima at 566 nm and 558 nm as also shown in fig. 1b. A mirror image of the spectrum is obtained for the *b* cytochromes oxidized by uncoupler addition (C–B) and this transition is thus a reversible, energy dependent change. When dithionite is then used to reduce the remaining *b* cytochrome in the uncoupled mitochondria (D–C), the spectrum is identical in shape but of slightly greater absorbance than the energy dependent change.

### 3.3. *The reduced-oxidized difference spectra of the b cytochromes of pigeon heart mitochondria at 77° K*

The spectra were measured by the trapped steady state technique of Chance and Schoener [2] with the samples at 77° K. The pigeon heart mitochondria were supplemented with KCN, ascorbate, TMPD and rotenone, and spectrum A (fig. 3) was obtained by freezing an aliquot before (reference) and two minutes after (measure) the addition of 5 mM succinate. The *b* cytochrome reduced by the succinate has

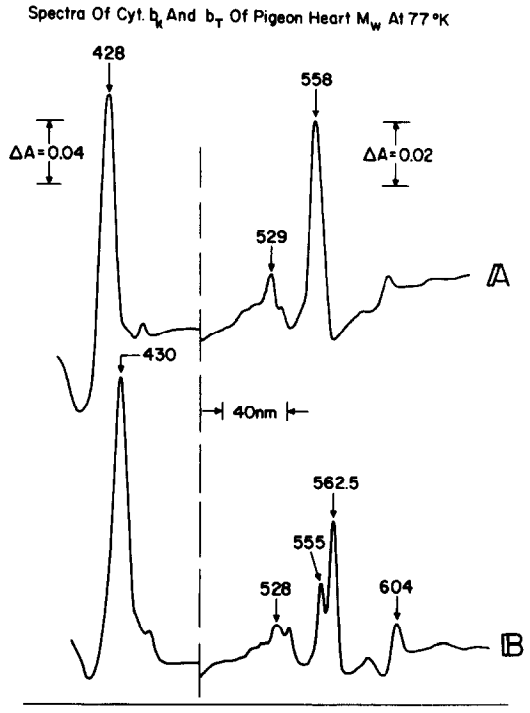


Fig. 3. The absorption spectra of *b* cytochromes in pigeon heart mitochondria at 77° K. The $PHM_W$ were suspended at 5.8 mg protein/ml and supplemented with 2.5 μM rotenone, 5 mM KCN, 5 mM ascorbate and 80 μM TMPD. Spectrum A: The reference sample was withdrawn and injected into a spectrophotometer cuvette which had been precooled to liquid nitrogen temperature. The measure sample was similarly treated but was withdrawn 2 min after the addition of 5 mM succinate. Spectrum B: The mitochondrial suspension was supplemented with 5 mM succinate and the reference sample withdrawn 2 min later. The measure sample was withdrawn after the further addition of 1.2 mM ATP.

a symmetric single alpha band with a maximum at 558 nm, a beta band with a maximum at 529 nm and a Soret band at 428 nm. The spectrum of the remaining *b* cytochrome was obtained by freezing an aliquot of the mitochondrial suspension 2 minutes after the addition of succinate (reference) and after the addition of succinate and 1.2 mM ATP (measure). The *b* cytochrome reduced on addition of ATP is shown as curve B in fig. 3. This cytochrome has a double alpha band with maxima at 562.5 nm and 555 nm, a complex beta band at 535 nm and 528 nm and a single Soret band at 430 nm.

## 4. Discussion

The mitochondrial respiratory chain has been shown to contain two spectrally different *b* cytochromes which respond quite differently to reduction by substrate and to the energy state of mitochondria. One of these cytochromes at room temperature has a single symmetric alpha band at 561 nm; at liquid nitrogen temperature it has a single symmetric alpha band at 558 nm, a beta band at 529 nm and a Soret band at 428 nm. This cytochrome is readily reduced by succinate in both coupled and uncoupled mitochondria. The other *b* cytochrome at room temperature has a double alpha band at 565 nm and 558 nm; at liquid nitrogen temperature it has a double alpha band at 562.5 nm and 555 nm, a complex beta band at 535 nm and 528 nm and a Soret band at 430 nm. This cytochrome is readily reduced by succinate in mitochondria in the presence of ATP but not in uncoupled mitochondria.

These properties of the two *b* cytochromes clearly identify the cytochrome having an alpha band at 561 nm as the higher potential *b* cytochrome ($E_m$ = + 30 mV) of Wilson and Dutton [8]. The *b* cytochrome having a double alpha band has properties identifying it with low potential *b* cytochrome (cytochrome $b_T$, $E_m$ = − 30 mV) of Wilson and Dutton which they have reported to have an energy dependent oxidation–reduction midpoint potential (in the presence of ATP, the $E_m$ = + 245 mV).

The addition of ATP to succinate-reduced mitochondria causes an energy dependent reduction of cytochrome $b_T$ which is reversed by uncoupler. Subsequent reduction by dithionite reduces cytochrome $b_T$ under low energy conditions (uncoupled mitochondria) and yet the observed spectral change is indistinguishable from the energy dependent change. Therefore, it can be concluded that both low and high potential forms of cytochrome $b_T$ have the same spectra. The low midpoint potential of cytochrome $b_T$ in uncoupled mitochondria (low energy) readily explains the failure of succinate to completely reduce the *b* cytochromes, which has been reported by several investigations [4, 17, 18].

In one case [6], a double alpha band was observed in an energized-non-energized difference spectrum (ref. 6, fig. 1b). Furthermore, preliminary evidence of an energy dependent cytochrome response was obtained in the Soret band (ref. 2, figs. 5 and 6). Thus these data extend those obtained previously and serve to explain the more recent data of Slater and his coworkers [11, 12].

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