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THE SPECTRAL PROPERTIES OF THE *b* CYTOCHROMES IN INTACT MITOCHONDRIA

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SUMMARY

The mitochondrial respiratory chain of pigeon heart has been shown to contain two spectrally and functionally different *b* cytochromes. One of these *b* cytochromes (b_K) at room temperature has a single symmetrical α band at 561 nm; at liquid nitrogen temperature it has a single symmetrical α band at 558 nm, a β band at 529 nm a Soret band at 428 nm. This cytochrome is readily reduced by succinate in both coupled and uncoupled mitochondria.

The second cytochrome (b_T) at room temperature has a double α band at 565 nm and 558 nm; at liquid nitrogen temperature it has a double α band at 562.5 nm and 555 nm, a β band at 535 nm and 528 nm, and a Soret band at 430 nm. This cytochrome *b* is readily reduced by succinate in mitochondria in the presence of ATP but not in uncoupled mitochondria.

Potentiometric titration identifies cytochrome b_K as the higher potential *b* cytochrome of D. F. WILSON AND P. L. DUTTON (*Biochem. Biophys. Res. Commun.*, 39 (1970)59), and the latter as the lower potential *b* cytochrome.

The spectrum of reduced cytochrome b_K shifted slightly to longer wavelength when antimycin A is added, but antimycin has no measurable effect on the spectrum of reduced cytochrome b_T.

INTRODUCTION

A direct role of cytochrome *b* in mitochondrial electron transport and energy conservation was first postulated by CHANCE *et al.*[1,2] and by SLATER *et al.*[3]. These postulates were based on the anomalous kinetic[4,5] and spectroscopic[2–7] behavior of the *b* cytochrome in submitochondrial particles and to a lesser extent in intact mitochondria. Recently, WILSON AND DUTTON[8] have used potentiometric measurement of the oxidation–reduction midpoint potentials of the cytochromes to obtain

Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulfate; PES, phenazine ethosulfate.

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evidence for the existence of two chemically distinct species of *b* cytochromes in rat liver[8] and pigeon heart[9] mitochondria and of three chemically distinct species in beef heart mitochondria[10]. In rat liver and pigeon heart mitochondria they observed that one of the two *b* cytochromes (b_T) has an energy dependent oxidation–reduction midpoint potential and postulated that it directly participates in the transduction of electrical potential of electron transport to chemical potential suitable for ATP synthesis. Evidence has also been obtained by CHANCE *et al.*[9,11] for the existence of two kinetically distinct species of *b* cytochrome in pigeon heart mitochondria. They have reported that these two species have different α absorption maxima based on spectra obtained by "time resolution" of their kinetic curves.

It is the purpose of this communication to present the spectral properties of the two *b* cytochromes of pigeon heart mitochondria and to examine the previously reported properties of cytochrome *b* in the context of its being not one but two different cytochromes. Such a report is timely because of the need to characterize these two cytochromes and to provide a proper prospect for the reports of an energy dependent shift in the spectrum of reduced cytochrome *b*[12,13].

MATERIALS AND METHODS

Pigeon heart mitochondria were prepared in 0.225 M mannitol–0.075 M sucrose–0.2 mM EDTA, pH 7.4, according to the method of CHANCE AND HAGIHARA[14]. 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[15]. This spectrophotometer is equipped with Bausch and Lomb 250 mm monochomator with a 600 line per mm grating. Repeatability and reproducibility of the position of cytochrome peaks were $\pm$ 5 Å. Measurements of the steady state absorbance change of cytochromes were made with an Aminco–Chance dual wavelength spectrophotometer and a Perkin–Elmer 356 two wavelength spectrophotometer.

Potentiometric measurements of cytochromes were performed in the apparatus described by DUTTON[16] which permitted the control of oxidation–reduction–potential of biological suspension under strict anaerobic conditions and anaerobic sampling. Oxidation–reduction mediators were those used by WILSON AND DUTTON[8,17], namely 25 μM tetramethyl-*p*-phenylenediamine (TMPD), 25 μM diaminodurol, 50 μM phenazine methosulfate (PMS), 50 μM phenazine ethosulfate (PES), 15 μM duroquinone and 50 μM 2-hydroxynaphthaquinone in measuring the cytochrome *b* region. At the desired oxidation–reduction potential a positive pressure of argon gas was used to force a sample of the mitochondrial suspension through a Hamilton Teflon needle into a spectrophotometer cuvette precooled to liquid nitrogen temperature.

Antimycin A and rotenone were added as small volumes (1–2 μl in 3 ml) of ethanolic solutions. Antimcyin A solution was standardized by absorption measurements at 320 nm[18]. TMPD and L-ascorbic acid were products of Sigma Chemical Co. and were used in aqueous solutions. The ascorbate solution was neutralized to pH 6.8 with KOH.

Protein was determined by the biuret method[29] with bovine serum albumin as standard.

RESULTS

The spectral properties of the low potential cytochrome b

As shown in Fig. 1, when a suspension of pigeon heart mitochondria is supplemented with succinate and glutamate respiration is initiated and the *b* cytochromes (as measured at 562.5 nm *minus* 575 nm) become partially reduced. In time the oxygen in the suspending medium is exhausted and the aerobic to anaerobic transition gives an increased reduction which displays a slight overshoot and then stabilizes with the *b* cytochromes approximately 60 to 75 % reduced, depending on the exact experimental conditions. Additions of a limiting amount of ATP causes a reduction of the cytochromes which is reversed if the ATP is completely hydrolized or if uncoupler is added. Dithionite addition completely reduces the *b* cytochromes, after which the antimcyin A causes almost no spectral change at this wavelength pair.

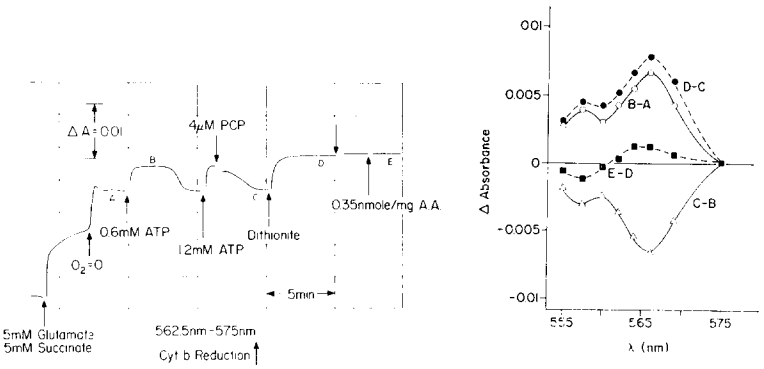

Fig. 1. The effect of substrate and ATP on the degree of reduction of *b* cytochromes in pigeon heart mitochondria. The pigeon heart mitochondria were suspended at 2.7 mg protein/ml in a medium containing 0.225 M mannitol, 0.07 M sucrose, 2 mM EDTA and 50 mM Tris–HCl, pH 7.4. The reagents were added at the indicated times and final concentrations. A.A., antimycin A; PCP, pentachlorophenol.

Fig. 2. The spectral properties of the *b* cytochromes in pigeon heart mitochondria. The pigeon heart mitochondria were suspended and treated as given in the legend of Fig. 1. A series of experiments were carried out in which the measure wavelength was varied but the reference wavelength was left at 575 nm. (I) The absorbance changes obtained on transition from the mitochondria in the absence of substrate to the time designated by the letters A, B, C, D, and E (Fig. 1) are plotted as a function of the wavelength of the "measuring" light. (II) The difference in absorbance between specified spectra shown in part I are obtained directly by plotting the absorbance changes designated in Fig. 1. Therefore, Curve B—A represents the spectrum of the *b* cytochrome which is reduced when ATP is added to anaerobic mitochondria in the presence of glutamate and succinate. C — B, the *b* cytochrome oxidized when the uncoupler pentachlorophenol is added to the ATP treated mitochondria. D — C, the *b* cytochrome reduced when dithionite is added to uncoupled mitochondria in the presence of glutamate and succinate. E — D, the spectral shift induced in the *b* cytochrome spectrum by the addition of antimycin A to mitochondria to which dithionite has been added as a reductant.

Several experiments were carried out under identical conditions except that the measuring wavelength was varied while the reference wavelength was held constant at 575 nm. The differences in absorbance between the points designated A through E (Fig. 1) were then plotted as a function of the measuring wavelength (Fig. 2). The spectrum of the *b* cytochrome reduced by ATP addition (B–A) has two maxima, one at 566 nm and the other at 558 nm. A mirror image of the spectrum is

obtained for the *b* cytochrome oxidized by hydrolysis of ATP or 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.

The effect of antimycin A on the spectra of the dithionite reduced b cytochromes of pigeon heart mitochondria[9]

The addition of a saturating amount of antimycin A (0.7 nmole/mg protein) to the mitochondrial suspension after complete reduction by dithionite (Fig. 1) causes a shift in the absorption maxima of the *b* cytochromes, as shown in Fig. 2 (E–D). This is clearly a spectral shift with a negative trough at 558 nm and a positive maximum at 564 nm. This difference is very similar to that reported by PUMPHREY[20] for the effect of antimycin A on the dithionite reduced *b* cytochrome in beef heart submitochondrial particles. The spectra shift is very small, as evidenced by the fact that the peak and trough represent less than 8 % of the cytochrome *b* absorbance. When very small shifts are involved, the point of zero absorbance change approaches the maximum of the absorption band which shifted. Thus, the antimycin A induced absorbance shift arises from an absorption band with a maximum near 561 nm.

The effect of antimycin A on the ATP induced cytochrome b reduction

The mitochondrial suspension was treated with KCN and the *c* cytochromes reduced by adding ascorbate *plus* TMPD in order to eliminate cytochromes *c* and c_1 contribution[4,21]. As shown in Fig. 3, the addition of succinate causes a partial reduction (60 %) of the *b* cytochromes as measured at 562 nm *minus* 575 nm. This reduction is increased to approximately 90 % when the ATP is added. Antimycin A at 0.43 nmole/mg protein induces a strong cyclic oxidation of the cytochrome(s) *b* which has a maximum in about 2 min. This oxidation is followed by reduction which leads to essentially 100 % reduction of the *b* cytochromes in approximately 20 min. The spectra of the *b* cytochromes were measured by carrying out identical experiments but varying the measuring wavelength. In Fig. 4-I, the spectra are plotted as the absorbance change between the mitochondria treated with KCN, ascorbate and TMPD and the points designated on Fig. 3 as A–D. The *b* cytochrome reduced by succinate addition (A) has a maximum at 561 nm and this increases in absorbance and the maximum shifts to 565 nm on addition of ATP (B). The absorption spectra are also plotted for 2 min after antimycin A addition (C) and for 20 min after antimycin A addition (D). The species of *b* cytochrome involved in each transition can be more readily determined from the difference spectra given in Fig. 4-II. Curve A is repeated from Fig. 4-I for comparison. It is apparent that the cytochrome *b* reduced by succinate (A) has a different spectrum from that reduced by ATP addition (B–A). The former has an absorption band with a single maximum at near 561 nm, while the latter has an absorption band with two maxima, one at 565 nm and the other at 558 nm. The *b* cytochrome oxidized by antimycin A addition (C–B) appears to be a mixture of the two spectrally different forms (but dominantly the one having double α maxima) with the antimycin A induced shift superimposed on the oxidation–reduction difference. The spectrum D–B shows a trace of cytochrome *b* reduction and the antimycin A induced spectral shift.

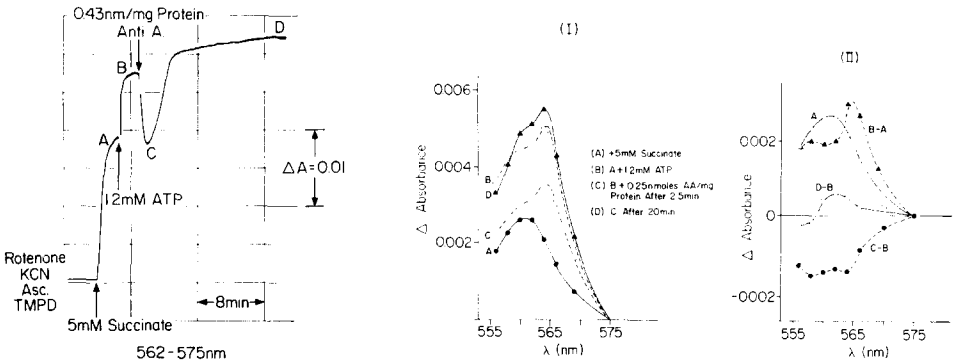

Fig. 3. The effect of the addition of antimycin A (Anti. A.) on the degree of reduction of the *b* cytochromes of pigeon heart mitochondria. The pigeon heart mitochondria were suspended in the assay medium at 3.65 mg protein/ml. They were then supplemented with 2.5 μM rotenone, 5 mM KCN, 5 mM sodium ascorbate (Asc.) and 80 μM TMPD. The reagents were added at the indicated times and final concentrations.

Fig. 4. The effect of antimycin A (AA) on the spectra of the *b* cytochromes in pigeon heart mitochondria. The pigeon heart mitochondria were suspended at 1.1 mg protein/ml and treated as given in the legend of Fig. 3. A series of experiments were carried out in which the wavelength of the measuring light was varied but the wavelength of the reference light was 575 nm. (I) The absorbance changes obtained on transition from the mitochondria in the presence of rotenone, KCN, ascorbate and TMPD (see legend Fig. 3) to the position designated by the letters A, B, C, and D (Fig. 3) are plotted as a function of the wavelength of the measuring light. (II) The difference in absorbance between the specified spectra in (I) are obtained by plotting the absorbance changes designated in Fig. 3. Therefore Curve B — A represents the spectrum of the cytochrome which is reduced when ATP is added to mitochondria in the presence of succinate and rotenone. C — B, the spectrum of the *b* cytochrome which is oxidized 2.5 min after the addition of antimycin A. D — B, the difference between the spectra of the *b* cytochromes reduced in the presence of succinate, ATP and antimycin A 20 min after the addition of antimycin A.

The reduced minus 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. 5) was obtained by freezing an aliquot before (reference) and 2 min after (measure) the addition of 5 mM succinate. The *b* cytochrome reduced by the succinate has a symmetric single α band with a maximum at 558 nm, β 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 min 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. 5. This cytochrome has a double α band with maxima at 562.5 nm and 555 nm, a complex β band at 535 nm and 528 nm and a single Soret band at 430 nm. In each case, of course, these values are for the reduced *minus* oxidized difference spectra and the absorption maxima are only approximately the same as for the absolute spectrum of the reduced cytochrome.

The potentiometric measurements of b cytochromes

Potentiometric titration of the reduction of the *b* cytochromes was performed

as described by WILSON AND DUTTON[8,17]. At the desired oxidation–reduction potential samples of the mitochondrial suspension were anaerobically transferred to a cuvette precooled to 77°K. The difference spectra of cytochromes thus obtained at 77°K are shown in Fig. 6. For each spectrum the reference sample is an aerobic mitochondrial suspension while the measure sample is an anaerobic mitochondrial suspension at the indicated oxidation–reduction potential. For coupled mitochondria in the presence of ATP, as the oxidation–reduction potential is progressively lowered the *b* cytochrome having α maxima at 562.5 nm and 555 nm is first reduced and then the *b* cytochrome having an α maximum at 558 nm (Fig. 6A–D). The order of reduction is reversed in uncoupled mitochondria (Fig. 6E–H). The oxidation–reduction potential required to reduce the *b* cytochrome having α maxima at 562.5 nm and 555 nm is much more positive in coupled mitochondria in the presence of ATP than it is in the uncoupled mitochondria. No change is evident in the potential required to reduce the cytochrome having an α maximum at 558 nm.

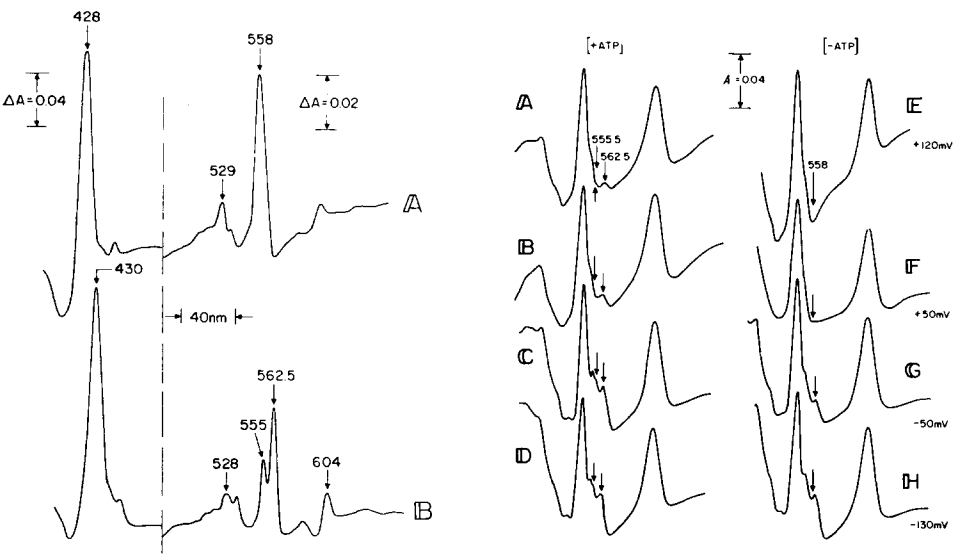

Fig. 5. The absorption spectra of cytochromes b_K and b_T in pigeon heart mitochondria at 77°K. The pigeon heart mitochondria were suspended at 5.8 mg protein/ml and supplemented with 2.5 μM rotenone, 5 mM KCN, 5 mM ascorbate and 80 μM TMPD. Spectrum A: The reference sample was withdrawn and injected 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.

Fig. 6. Low temperature difference spectra showing the correlation between the oxidation–reduction potential of the mitochondrial sample before freezing and the *b* components in pigeon heart mitochondria. Pigeon heart mitochondria were suspended at 2.7 mg protein/ml in a medium containing 0.225 M mannitol, 0.07 M sucrose, 2 mM EDTA and 50 mM Tris–HCl, pH 7.4. 2.5 μM rotenone and indicated redox mediators were added. Potentiometric titration of the anaerobic suspension was carried out according to the method of WILSON AND DUTTON[8]. At the potential as indicated, the sample was anaerobically transferred to a cuvette at 77°K immediately frozen to 77°K and then the difference spectrum was taken using the oxidized sample as a reference. Curves A–D, 1.2 mM ATP was added about 30 sec before removing the sample from the mitochondrial suspension. The oxidation–reduction potentials of the mitochondrial suspension before sampling are indicated. Curves E–H, without any addition.

This behavior is in agreement with the report of WILSON AND DUTTON[8] that one of the two *b* cytochromes (cytochromes b_T) in rat liver and in pigeon heart mitochondria[9] has an energy dependent midpoint potential (−30 mV uncoupled and +245 mV with excess ATP) but the other (cytochrome b_K) does not.

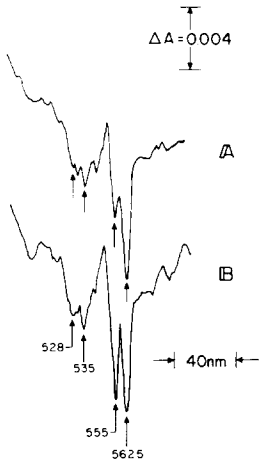

Fig. 7. The oxidized *minus* reduced absorption spectra of the *b* cytochrome oxidized when antimycin A or uncoupler are added to mitochondria in the presence of succinate and ATP. The mitochondria were suspended in 2.1 mg protein/ml and supplemented with 2.5 μM rotenone, 5 mM KCN, 5 mM ascorbate, 80 μM TMPD, 5 mM succinate and 1.2 mM ATP. Spectrum A: The reference sample was withdrawn and frozen. The measure sample was frozen 2 min after the addition of 20 μM dicumarol. Spectrum B: The reference sample was withdrawn and frozen. The measure sample was frozen 2 min after the addition of 0.3 nmole antimycin A per mg mitochondrial protein.

The oxidized minus reduced spectra of the b cytochrome oxidized when uncoupler or antimycin A are added to succinate and ATP treated mitochondria

When mitochondria are treated with succinate and ATP, the addition of either uncoupler (Fig. 7A) or antimycin A (Fig. 7B) causes an oxidation of part of the *b* cytochrome. Spectra of the changes at room temperature (Figs. 2 and 4-II) reveal the uncoupler dependent change to be entirely the oxidation of the *b* cytochrome with a double α band, but the antimycin A dependent change is more complex. The spectra were, therefore, measured in samples at liquid nitrogen temperature to take advantage of the increased resolution at low temperature[22,23]. As may be seen in Fig. 7, the spectra are remarkably similar. Thus, the same *b* cytochrome is oxidized by antimycin A addition as is oxidized by uncoupler addition. This is consistent with the observation that antimycin A prevents the ATP induced midpoint potential change in cytochrome b_T (P. L. DUTTON, D. F. WILSON AND M. ERECINSKA unpublished results).

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 the mitochondria. One of these *b* cytochromes at room temperature has a single symmetric α band at 561 nm; at liquid nitrogen temperature it has a

single symmetric α band at 558 nm, a β 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 α band at 565 nm and 558 nm; at liquid nitrogen temperature it has a double α band at 562.5 nm and 555 nm, a double β 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.

The properties of the two *b* cytochromes clearly identify the cytochrome having an α band at 561 nm as the higher potential *b* cytochrome ($E_m = +30$ mV) of WILSON AND DUTTON[8,9]. Cytochrome which has also been called cytochrome b_K in honor of Prof. David Keilin. The *b* cytochrome having a double α band has properties identifying it with the low potential *b* cytochrome ($E_m = -30$ mV) of WILSON AND DUTTON[8,9] which they reported to have an energy oxidation–reduction midpoint potential (in the presence of ATP the $E_m = +245$ mV) and which they have proposed is part of the energy transducing mechanism at phosphorylation Site II. This cytochrome also has been designated cytochrome b_T. For the purposes of this discussion the cytochrome b_K and b_T nomenclature will be used.

The spectra of reduced cytochrome b_K is shifted to longer wavelengths when antimycin A is added, but the antibiotic has no measurable effect on the spectrum of cytochrome b_T. The spectral shift for cytochrome b_K is very small, but its behavior is similar to the shift reported by PUMPHREY[20] in dithionite reduced submitochondrial particles. There is no detectable antimycin A induced shift in the absorption maxima of either cytochromes b_K or b_T except the shift readily observed in dithionite reduced mitochondria. All other antimycin A induced absorbance changes appear to result from oxidation or reduction of the cytochrome b_T. The spectra (Figs. 2 and 4-II) and oxidation–reduction midpoint potentials[6] of the cytochromes b_K and b_T are such that the wavelength pair (566 nm *minus* 560 nm) used by BRYLA *et al.*[24] and SLATER *et al.*[12,13] would be expected to give a maximal sensitivity to differential oxidation–reduction of cytochromes b_K and b_T.

The addition of ATP to succinate reduced mitochondria causes an energy dependent reduction of cytochrome b_T which is reversed by uncouplers.

Subsequent complete reduction by dithionite reduced cytochrome b_T under low energy conditions (uncoupled mitochondria) and yet the observed spectra change is indistinguishable from the energy dependent change. If there is an ATP induced change in the spectrum of either reduced cytochrome b_K or b_T, the change is extremely small. The low midpoint potential of cytochrome b_T in uncoupled (low energy) mitochondria of -30 mV readily explains the failure of succinate (midpoint potential $+30$ mV) to completely reduce the *b* cytochromes[4,25,26].

The experimental data from potentiometric analysis[8,9], kinetic analysis[9,11] steady state absorbance changes[20,27] and the spectral data in this paper can be fitted together if the antimycin A and ATP dependent changes measured by the wavelength pair 566 nm *minus* 560 nm reported by SLATER *et al.*[12,13,24] are the result of oxidation and reduction of the cytochromes designated here as b_K and b_T. This interpretation suggests that the addition of antimycin A or ATP to substrate reduced mitochondria or submitochondrial particles causes a reduction of the long wavelength cytochrome b_T (apparent red shift). The antimycin A could accomplish this by lowering the effec-

tive oxidation–reduction potential expressed on the *b* cytochromes by the substrate. ATP, on the other hand, could make the cytochrome b_T more easily reducible at the same oxidation–reduction potential by causing its midpoint potential to become more positive[8,9].

CHANCE *et al.*[2,6,7] observed a novel form of cytochrome *b*, the properties of which were dependent on the energy state of mitochondria. This *b* cytochrome (cytochrome b_{555}) had absorption maxima at 555 nm and 425 nm at 77°K. The observed double α band of cytochrome b_T may resolve the difference between cytochrome b_{555} of CHANCE *et al.* and the cytochrome absorbing at 565 nm of SLATER *et al.*[12,13].

The double α band of cytochrome b_T is particularly interesting because the split is greater than has previously been observed. Cytochrome b_5 has a double α band, but the separation of the maxima are only 5 nm at 77°K, considerably less than the 7.5 nm found for cytochrome b_T. KEILIN[28] has reported greater separation of the "twin" α bands of the reduced whale myoglobin hydrazine and nicotinate compounds. She has attributed this four banded structure of the absorption structure to a molecular species in which the heme Fe–ligand bond is asymmetric, *i.e.* the ligand is sterically forced out of its preferred alignment. This is a very reasonable explanation of the unusual spectrum of cytochrome b_T.

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