SPECTROSCOPIC EVIDENCE FOR TWO *b* CYTOCHROMES IN MITOCHONDRIA OF INTACT EHRLICH ASCITES TUMOR CELLS

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1. Introduction

Wilson and Dutton [1] have reported the existence of two chemically distinct forms of cytochrome *b* in the respiratory chain of rat liver mitochondria. One of these forms has its midpoint potential dependent on energy, and it has been designated cytochrome b_T (energy-transducing cytochrome *b*) so that it can be distinguished from the Keilin's cytochrome *b* (cytochrome b_K) [2]. These two forms of cytochrome *b* have been described also in pigeon heart [2] and beef heart [3] mitochondria. Previous and more recent kinetic data [2, 4] have allowed to establish that cytochrome b_T is primarily involved in energy conservation at site II, while cytochrome b_K serves exclusively as an electron carrier. The two *b* cytochromes can be also distinguished spectroscopically in reduced *minus* oxidized difference absorption spectra. In pigeon heart mitochondria the transducing form has the maximum shifted at a longer wavelength with respect to the other form [2]. Detailed information on the spectral properties of *b* cytochromes at room- and low-temperature in pigeon heart mitochondria have been given [5, 6]. No evidence has yet been presented on two forms of cytochrome *b* in whole mammalian cells, except for the *b* and *b*(d) cytochromes described by Gonse [7] in spermatozoa. In this case, however, the energy depence of one of the two species has not been shown.

In the present communication we report the existence of two spectroscopically different forms of cytochrome *b* in the mitochondria of intact Ehrlich ascites tumor cells. It has been possible to make such a distinction by the use of an uncoupling agent which causes oxidation of only one of the two forms of cytochrome *b*. The percent contribution of each form to the total cytochrome *b* content corresponds to that found for this pigment in mitochondria isolated from normal tissues [3]. Thus the intimate details of energy coupling in ascites cell mitochondria are similar to those of other mitochondria.

2. Experimental

Ehrlich-Lettre' hyperdiploid ascites tumor cells were grown in albino ICR mice by weekly transfer of 0.2 to 0.3 ml of ascites fluid. The cells, harvested 6 to 8 days after the inoculation, were washed once in a saline phosphate medium. After washing, the cells were suspended in the same medium at the final concentration of 50–60 mg dry weight/ml. The medium had the following composition: 154 mM NaCl, 6.2 mM KCl and 11 mM sodium phosphate buffer, pH 7.4 [8].

Cytochrome kinetics and room-temperature difference absorption spectra were performed in the dual wavelength/split beam Aminco-Chance spectrophotometer. Low-temperature spectra were performed in a Johnson Foundation split-beam spectrophotometer [9, 10].

The 4,5,6,7-tetrachloro-2-trifluoromethylbenzi-

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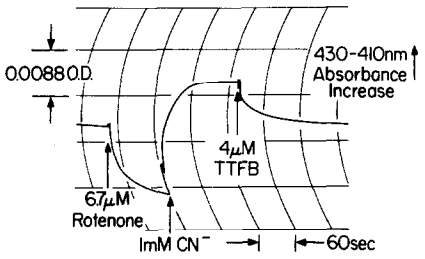

Fig. 1. Oxidation–reduction changes of cytochrome *b* in Ehrlich-Lettre' ascites tumor cells. The cells were suspended in a saline phosphate medium (pH 7.4) (for composition see Experimental) at the final concentration of 6.65 mg dry weight/ml. Room temperature, endogenous substrates.

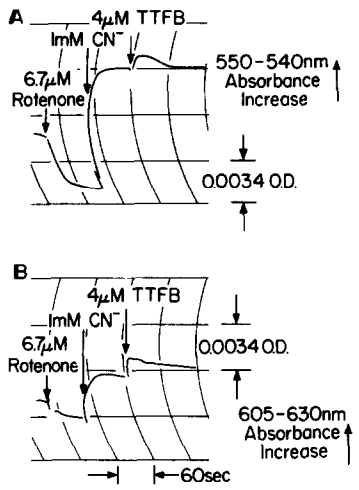

Fig. 2. Spectrophotometric recordings of redox changes of cytochromes $c + c_1$ (A) and $a + a_3$ (B). The cell suspension concentration was 6.18 mg dry weight/ml. For other conditions see fig. 1.

midazole (TTFB) was kindly supplied by Dr. R.B. Beechey of Shell Research, Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent (Great Britain). Rotenone and Antimycin A were purchased from Sigma Chemical Co. All other chemicals were products of J.T. Baker Chemical Co.

3. Results and discussion

Figs. 1 and 2 show the effect of the uncoupler

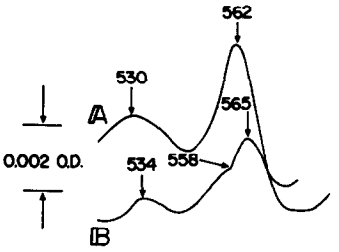

Fig. 3. Room-temperature difference absorption spectra of *b* cytochromes in ascites tumor cells. (A) cells (10.8 mg dry wt./ml) were treated with 6.7 μM rotenone and 4 μM TTFB and a baseline was recorded. 7 μg/ml antimycin A was then added to the measuring cuvette and the difference spectrum was recorded. (B) cells (6.84 mg dry wt./ml) were treated with 1 mM sodium cyanide and a baseline was recorded. 4 μM TTFB was then added to the reference cuvette and the difference spectrum recorded. Other conditions are as described in fig. 1.

TTFB on the steady state of some electron carriers in the inhibited mitochondrial respiratory chain of intact Ehrlich-Lettre' ascites tumor cells. Fig.1 presents kinetics of cytochrome *b* measured at 430–410 nm. Addition of 6.7 μM rotenone to aerobic cells utilizing endogenous substrates causes an oxidation of cytochrome *b* corresponding to 11.0 nmoles/g dry weight. This oxidation is due to the fact that in these cells cytochrome *b* is partially reduced in the aerobic steady state [7, 11]. Further addition of 1 mM sodium cyanide completely reduces cytochrome *b* through the rotenone leak. The reduction induced by cyanide corresponds to 18.3 nmoles/g dry weight and gives the titration of the total amount of cytochrome *b* reducible by endogenous substrates in these cells. The sequence of additions shown above indicates that 60% of cytochrome *b* is reduced in the aerobic steady state. Addition of 4 μM TTFB to rotenone- and cyanide-inhibited cells causes reoxidation of 40% of the total amount of cytochrome *b* reduced by cyanide, in about 3 min. The effect of TTFB distinguishes two portions of cytochrome *b* in these cells. Only one of them is sensitive to the energy state of mitochondria, as indicated by its oxidation with a decrease of the phosphate potential induced by the uncoupler. The amount of cytochrome *b* oxidized by TTFB could correspond to the high energy form of cytochrome b_T which becomes reduced in the presence of cyanide. As indicated by the

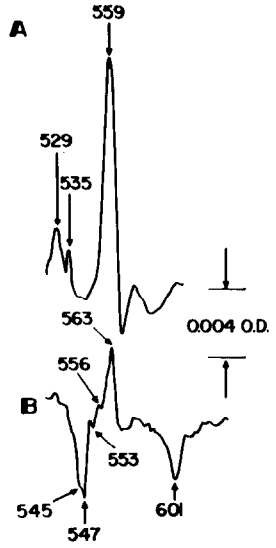

Fig. 4. Low-temperature (77°K) difference absorption spectra of *b* cytochromes in ascites tumor cells. (A) cells (27.4 mg dry wt./ml) were treated with 13 μM rotenone and 2.7 μM TTFB and divided in two cuvettes. 20 μg/ml antimycin A was then added to the measuring cuvette. The samples were chilled in liquid nitrogen and the difference spectrum recorded. (B) cells (31.0 mg dry wt./ml)were treated with 13 μM rotenone and 1.3 mM sodium cyanide. 1.6 μM TTFB was added to the reference cuvette and the samples chilled 30 sec after the addition of the uncoupler.

experiments shown in fig. 2 (A and B), the oxidation cytochrome *b* by TTFB corresponds to a re-equilibration of reducing equivalents through the mitocondrial respiratory chain in the absence of energy. Indeed, upon addition of the uncoupler a reduction of cytochrome $c + c_1$ (550–540 nm) and cytochrome oxidase (605–630 nm) may be observed. The more reduced steady state of these two cytochromes induced by the uncoupler is only transient (about 30 sec), probably because a leak of electrons through the cyanide-inhibited site or cytochromes interaction with substrate redox couples.

Following the indications of the kinetic experiments presented above, an attempt has been made to identify spectroscopically two distinct forms of cytochrome *b* in these cells. Fig. 3 shows room-temperature difference absorption spectra of intact cells performed with different combination of inhibitors and TTFB. In the spectrum A, the absorption change induced by antimycin A has been recorded between two samples treated aerobically with rotenone and TTFB. The spectrum of cytochrome *b*, thus obtained, shows α- and β-bands at 562 and 530 nm respectively. The spectrum B has been obtained by treating cells with cyanide and adding TTFB only to one sample. The reduced *minus* oxidized difference spectrum shows an α peak at 565 with a shoulder at 558 nm and a β-band at 534 nm. The spectra A and B show peaks which correspond to those obtained for the b_K and b_T cytochromes of pigeon heart mitochondria [5, 6]. A better distinction between these two forms of cytochrome *b* in Ehrlich ascites cells is given by the low-temperature difference absorption spectra presented in fig. 4. Spectrum A is the difference between cells treated with rotenone and TTFB in the presence and absence of antimycin A. This spectrum shows a single α peak at 559 nm and two maxima in the β region at 535 and 529 nm. The spectrum B has been obtained by adding rotenone and cyanide to both the samples and treating one of them for 30 sec with TTFB. As shown in fig. 2, the short period of treatment with the uncoupler allows to see also the transient redox changes of cytochrome $c + c_1$ and cytochrome oxidase, opposite to that of cytochrome *b* described in fig. 1. On the other hand, in these conditions, only a partial amount of cytochrome *b* (about 50% of that normally oxidized by TTFB) is evident. This accounts for the difference in absorption between the two forms of cytochrome *b* shown in fig. 4. Cytochrome oxidase shows a maximum at 601 nm; cytochrome c_1 is at 553 nm, while cytochrome *c* shows the α_1 (547 nm) and α_2 (545 nm) peaks. Cytochrome *b* shows a split α-band at 563 and 556 nm. Thus the 558 nm shoulder present at room-temperature can be resolved in the peak at 556 nm and distinguished from the cytochrome c_1 peak at 553 nm. Similar peak positions for cytochrome b_K and b_T at low-temperature have been described difference spectra of pigeon heart mitochondria [5, 6].

In conclusion, the use of an uncoupler and different inhibitors of the mitochondrial electron transport has given a tool for identifying two spectroscopically different forms of cytochrome *b* in the respiratory chain of intact Ehrlich ascites tumor cells. One form is still absorbing in uncoupled mitochondria and it has been evidenced by reduction in the presence of antimycin A. This form, which we identify with

the Keilin's cytochrome *b* (cytochrome b_K), has a single α-band at room- and low-temperature. The other cytochrome *b* form becomes reduced on energization of the mitochondrial respiratory chain and is oxidized in the presence of uncoupling agents. It shows typically a splitting of the α-band into two peaks at low-temperature. Thus, we consider this last form as the energy-transducing cytochrome *b* (cytochrome b_T), which has been shown to have an energy-dependent oxidation-reduction mid-point potential.

Acknowledgements

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