ATP AND pH INDUCED SPECTRAL CHANGES OF CYTOCHROME b IN RAT LIVER MITOCHONDRIA

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Summary. Addition of ATP to coupled rat liver mitochondria or mitochondrial fragments or a pH increase from 7.5 to 8.7 in uncoupled membranes induces the appearance of a spectrum of a b type cytochrome with peaks in the alpha region at 558 and 565 nm and in the beta region at 534 nm. The pH effect on the cytochrome b_{565} absorbance spectrum can be accounted for by the pH dependence of the redox potential of the cytochrome. The ATP effect may be mediated by a pH increase induced in the membrane during energy conservation.

The early suggestion of Chance and Williams(1) Chance and Schoener (2) and Slater and Colpa-Boonstra (3) of a direct role of cytochrome b in the reactions of energy conservation in mitochondria has been substantiated by more recent experimental results of Slater, Lee, Berden and Wegdam (4), Chance, Wilson, Dutton and Erecińska (5) and Wikström (6).

Two species of cytochrome b have been identified spectroscopically: a long-wavelength form, exhibiting a double alpha band with maxima at 558 and 565 nm, and a short wavelength form which exhibits a single alpha band at 562 nm. The reduction of the long-wavelength form is controlled by the energy coupling phenomenon. The short-wavelength form functions exclusively in electron transfer (7).

Thermodynamic differences have been described to be associated with the spectral differences of b cytochromes in mitochondria. The midpoint potentials of the cytochromes

b of the mitochondrial membrane in the uncoupled state have values of +20 and -35 mV, while in the energized state they are +20 and +240 mV (8). It has been suggested that the cytochrome b_{565} midpoint potential is shifted from -35 to +240 mV upon interaction with a ligand to form a species denoted $b_T \sim I$ (5) or $b_i^{3+} \sim X$ (4).

Alterations in the environment of the mitochondrial membrane such as pH changes (9) or potential changes (10) have been postulated to occur during energy conservation. Experimental data indicate that energy conservation is associated with changes in the membrane charge (11,12) and possibly in hydrophobicity (13) and potential (14). The redox potential of cytochrome b has been shown to be pH dependent between pH 7.0 and 8.5 (15) as well as that of ubiquinone (15).

This study was carried out in an attempt to investigate the effect of physical parameters on the reduction of the long-wavelength cytochrome b. It has been found that the appearance of a cytochrome b_{565} spectrum in rat liver mitochondria and mitochondrial fragments can be induced not only by ATP, as previously described (4,5,6,7), but also by a pH increase from 7.5 to 8.7 in the mitochondrial suspending medium, which contained the uncoupler, FCCP.

METHODS AND MATERIALS

Mitochondria and mitochondrial fragments from rat liver were prepared according to previously published methods (16,17).

All experiments were performed in a medium of the following composition: 0.25 M sucrose, 5 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 5 mM Na-glutamate, 5 mM Tris-succinate, 5 mM Na-ascorbate, 0.5 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), 3.3 mM NADH, 3 mM KCN and 3×10^{-6} M carbonyl cyanide-p-trifluormethoxy-phenylhydrazone (FCCP). When ATP was added to induce cytochrome b_{565} spectral changes, FCCP was omitted.

Absorption difference spectra were obtained in a split-beam

spectrophotometer (Johnson Research Foundation). Kinetics of absorbance changes were measured in a dual-wavelength spectrophotometer (Johnson Research Foundation), recorded in a Tektronix 564B storage oscilloscope and photographed.

pH was measured with a Beckman pH meter (Expandomatic).

Protein was determined by a biuret method (18). All reagents used were commercially available products. FCCP was a gift of Dr. Peter Heytler (Du Pont).

RESULTS

Effect of ATP on the spectrum of cytochrome b_{565}.

ATP, when added to a suspension of mitochondrial membranes, (supplemented with glutamate, succinate, NADH, ascorbate plus TMPD and KCN to keep the respiratory chain maximally reduced) induced an absorbance increase at 565 minus 575 nm as measured in a dual-wavelength spectrophotometer (Fig. 1A). The absor-

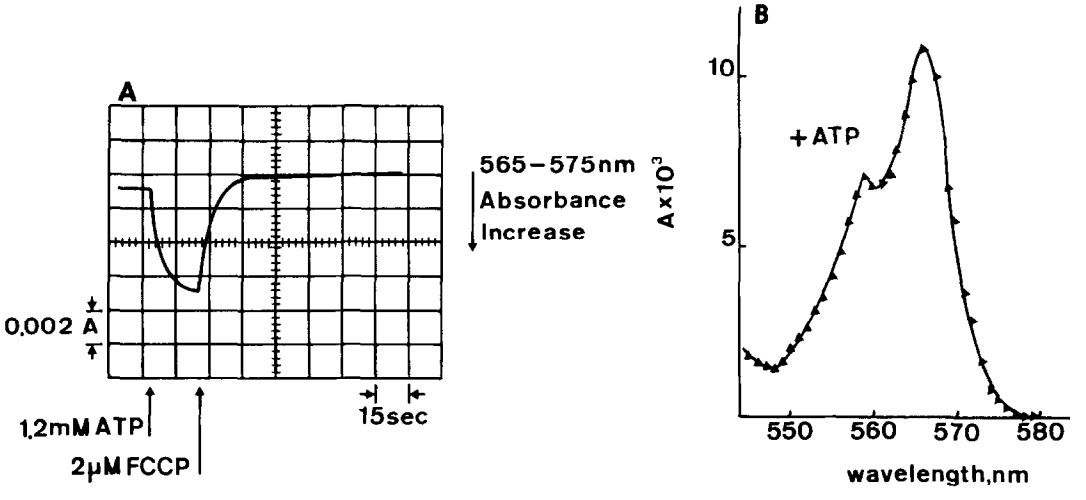

Fig. 1A. Kinetics of cytochrome b_{565} absorbance changes on addition of ATP. The incubation medium has been described in Methods and Materials. Mitochondrial protein was 6 mg per ml. The experiment was performed in a dual-wavelength spectrophotometer. B. ATP induced cytochrome b_{565} spectrum in rat liver mitochondria. The incubation medium was described in Methods and Materials. Protein concentration was 14 mg per ml. The spectrum was obtained in a split-beam spectrophotometer by adding to the sample cuvette 1.2 mM ATP. Reference cuvette was without ATP.

bance increase induced by ATP and interpreted as a reduction of a b type cytochrome (4,5,6,7) was reversed by the uncoupler, FCCP. Shown in Fig. 1B is an absorption difference spectrum obtained under conditions similar to those in Fig. 1A. ATP (1.2 mM) was present only in the sample cuvette. Two maxima (558 and 565 nm) and three minima (548, 560 and 578 nm) were observed. The ratio A_{558}/A_{565} was 1.5. The beta band was at 534 nm (not shown in the Figure).

Effect of pH on the spectrum of cytochrome b_{565}.

A pH transition from 7.5 to 8.5 was obtained (Fig. 2A) by adding sufficient amounts of 2N NaOH to a suspension of rat liver mitochondria under the same conditions of experiment reported in Fig. 1A. 3×10^{-6} M FCCP was present.

The absorbance increase of the suspension recorded with the dual-wavelength spectrophotometer at 565 minus 575 nm was approximately equal to that obtained in the absence of FCCP upon addition of ATP. The absorption change was reversed by addition of a roughly equivalent amount of HCl.

An absorption difference spectrum obtained under the conditions of the experiment shown in Fig. 2A is reported in Fig. 2B. The pH of the reference cuvette was 7.5, that of the sample cuvette, 8.7. The difference spectrum revealed two maxima, at 558 and 565 nm, and three minima, at 548, 560 and 578 nm respectively. The ratio A_{558}/A_{565} was 1.4. A beta band (not shown) with a maximum at 534 nm was also observed. The above characteristics identify the spectrum at pH 8.7 minus 7.5 as that of a b type cytochrome with a double alpha band indistinguishable from that induced by ATP (Fig. 1B, ref. 7; Fig. 2).

The pH dependence of the 565 nm absorbance is shown in Fig. 3. Absorbance changes following the addition of 2N NaOH to mitochondria suspended in the standard medium, initially at pH 6.5 and in the presence of FCCP, were followed with a dual-

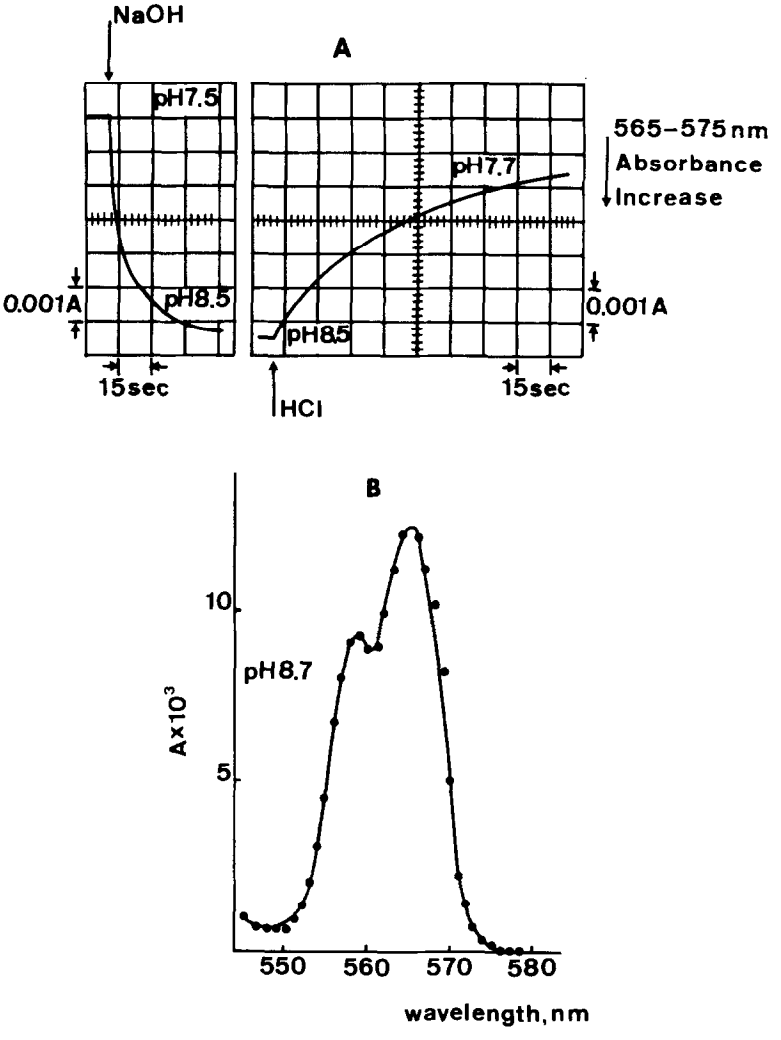

Fig. 2A. Response of cytochrome b_{565} absorbance to a pH transition. Experimental conditions as in Fig. 1A. The pH transition was obtained by adding about 15 microliters of 2N NaOH. Reversal was obtained by adding a slightly smaller amount of HCl. B. pH induced cytochrome b_{565} spectrum in rat liver mitochondria. Conditions as in Fig. 1B. 3×10^{-6} M FCCP was also present. The pH of the sample cuvette was 8.7, that of the reference 7.5.

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wavelength spectrophotometer. As can be seen, scarcely any absorbance change was observed below pH 7.1 and above pH 8.8. In experiments at pH 7.2, 7.9 and 9.4 in the absence of FCCP, ATP induced the absorbance increase reported in the graph as

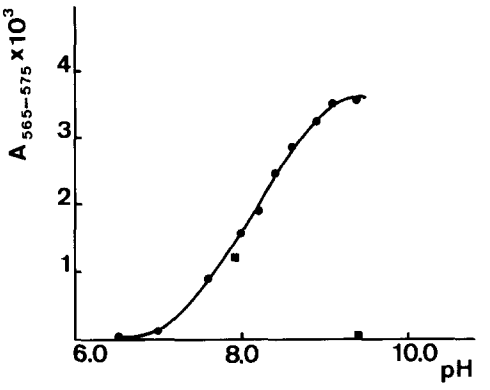

Fig. 3. pH dependence of cytochrome b_{565} absorbance in rat liver mitochondria. Experimental conditions as in Fig. 2A. Protein concentration was 6.2 mg per ml. 3×10^{-6} M FCCP was also present. The pH changes were obtained by adding 3 microliters of 2N NaOH to the 3 ml of mitochondrial suspension. Full squares represent 565 minus 575 nm absorbance changes induced by addition of 1.2 mM ATP at different pH values in the absence of FCCP.

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full squares. It appears that the ATP-induced absorbance change is roughly inversely proportional to the NaOH-induced change.

Effects of ATP and pH on the cytochrome b_{565} spectrum in mitochondrial fragments.

Similar experiments as those reported in Figs. 1 and 2 were carried out with mitochondrial fragments. The results are reported in Fig. 4. In the case of mitochondrial fragments, ATP and pH induced cytochrome b_{565} difference spectra were very similar. ATP was less effective than a pH transition in inducing the cytochrome b_{565} absorbance change. This was presumably due to the relatively low ATPase activity of the membrane fragments which were prepared in the presence of EDTA. The spectra of cytochrome b_{565} in mitochondrial fragments had characteristic maxima and minima similar to those of intact mitochondria. It should be noted that in mitochondria and mitochondrial fragments the pH induced changes were stable and not transient

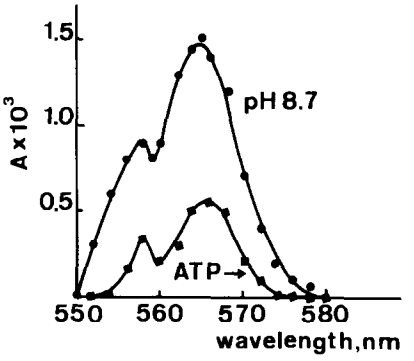

Fig. 4. ATP and pH induced cytochrome b_{565} spectra in rat liver mitochondrial fragments. Experimental conditions as in Fig. 1B. Fragments were equivalent to 3 mg per ml of protein. In the upper spectrum, the pH of the sample cuvette was 8.7 and that of the reference 7.5. In the lower spectrum 1.2 mM ATP was present in the sample cuvette. No ATP was present in the reference.

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as would be expected if they were the consequence of pH equilibration across the membrane.

DISCUSSION

The difference absorption spectrum at pH 8.7 minus 7.5 obtained in mitochondrial membranes supplemented with succinate, glutamate, NADH, ascorbate plus TMPD and cyanide was that of a cytochrome b type hemoprotein with a double alpha band very similar to the species observed on adding ATP to pigeon heart mitochondria (7) or rat heart mitochondria (4). The spectrum was also similar to the spectrum obtained by addition of antimycin A to mitochondrial membranes (4).

The difference absorption spectra in liver mitochondria, induced by ATP or by alkalinization of the medium, have been compared and no differences were detected within experimental error.

The pH induced cytochrome b spectrum (cytochrome b_{565}) was fully reversible between pH 6.5 and 9.5. It did not change with time and was similar in intact and fragmented mitochondria. These properties suggest that the appearance

of cytochrome b_{565} spectrum is a function of the pH of the medium rather than of proton equilibration across the mitochondrial membrane. A diffusion potential, produced by a pH difference across the membrane, would be expected to be transient.

At present, three possible explanations for the cytochrome b_{565} absorbance changes can be considered.

The appearance of cytochrome b_{565} following alkalinization of the membrane suspending medium can be simply interpreted as a consequence of a pH induced spectral shift of a ferrocytochrome b species in the membrane. The results presented in this study however can be more easily explained by assuming a pH dependence of cytochrome b_{565} redox potential according to the following reaction sequence, where cyt $b_{565\ ox}\cdot H^+$ and cyt $b_{565\ ox}$ denote a protonated and unprotonated form of ferricytochrome b_{565}, respectively.

$$\text{cyt } b_{565\ ox}\cdot H^+ \xrightleftharpoons[H^+ \text{ or FCCP}]{OH^- \text{ or ATP}} \text{cyt } b_{565\ ox} + H_2O \quad (1)$$

$$\text{cyt } b_{565\ ox} \longleftrightarrow \text{cyt } b_{565\ red} \quad (2)$$

At pHs higher than 7.0 (see Fig. 3) progressively larger amounts of the unprotonated form of ferricytochrome b_{565} would be available for reduction. The same dissociation of a proton from cyt $b_{565\ ox}\cdot H^+$ could be achieved by ATP or energy conservation. On the other hand, if the electron donor for cytochrome b_{565} had a redox potential sensitive to pH, effects qualitatively similar to those described above would be expected. A pH dependence of ubiquinone redox potential of -60 mV per pH unit has been described in mitochondria (15).

The identity between the cytochrome b_{565} spectrum obtained by adding ATP or alkalinization, suggests that an alkaline environment is created in the cytochrome b region by coupled ATP hydrolysis. Such a suggestion would be consistent with the finding that protons are released from

the membrane upon energization (10, 19).

Early changes of cytochrome b_{565} observed upon energy conservation in the mitochondrial membrane have been suggested to arise from the interaction between the hemoprotein and some unknown ligand to form the primary energy conserving intermediate (4, 5). On the basis of the observations reported here, it is attractive to suggest that early changes in cytochrome b_{565} occurring on energy conservation may arise from an increase in the pH of some part of the mitochondrial membrane. This pH increase may therefore represent one of the earliest events in energy conservation.

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