EFFECT OF pH ON THE REDOX STATE OF CYTOCHROME a IN ANAEROBIC, ATP-TREATED INTACT MITOCHONDRIA

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SUMMARY Addition of ATP to anaerobic, glutamate-reduced coupled mitochondria from rat liver or heart caused oxidation of cytochrome a having a peak at 608 nm. Subsequent increase in pH from 7.0 to 8.4 reversed the effect of ATP and subsequent decrease in pH from 8.4 to 7.0 induced reoxidation. This reversible effect of pH on the redox state of cytochrome a may reflect an electrochemical event in the energy-conserving mechanism of the terminal coupling site.

INTRODUCTION Wikström [1,2] reported that addition of ATP to anaerobic mitochondria supplemented with succinate caused considerable oxidation of cytochrome a having a peak at 607.5 nm. He concluded from his data that ATP decreases the apparent midpoint potential of cytochrome a by approximately 65 mV. Hinkle and Mitchell [3] explained the ATP-induced apparent decrease in the midpoint potential of cytochrome a as due to an electric potential difference between the hemes of cytochromes c and a, which is defined in the chemiosmotic hypothesis as a pH gradient between the suspending medium and matrix. Mitchell and Moyle [4] reported that protons are translocated outwards through the mitochondrial membrane during ATP hydrolysis in intact mitochondria. If this interpretation is correct, then it should be possible to decrease artificially the pH gradient in intact mitochondria formed by addition of ATP and the effect of ATP on the redox state of cytochrome a should be reversed by increasing the pH of the suspending medium. The present work showed that an increase in pH from 7.0 to 8.4 in anaerobic, ATP-treated intact

mitochondria from rat liver or heart caused reduction of cytochrome a and subsequent decrease in pH from 8.4 to 7.0 caused oxidation of cytochrome a. The possible explanation of the reversible effect of pH on the redox state of cytochrome a is discussed.

MATERIALS AND METHODS Intact mitochondria were isolated from rat liver by the method of Hogeboom [5], as described by Myers and Slater [6] and from rat heart by the method of Tyler and Gonze [7]. Protein was determined by the biuret method, as described by Cleland and Slater [8]. All reactions were carried out in medium containing 25 mM Tris-HCl buffer (pH 7.0), 50 mM sucrose, 5 mM $MgCl_2$, 2 mM EDTA and 15 mM KCl, with other components as indicated in the legends to figures, in a final volume of 3 ml. Measurements of absorbance changes of cytochrome a were made with a Hitachi, Model 356, two-wavelength spectrophotometer. Difference spectra were recorded with a Union, Model SM-401, High Sensitivity Split-beam Spectrophotometer equipped with a Union, Model SM-402, Baseline Corrector.

RESULTS AND DISCUSSION Fig.1(A) shows that reduction of cytochrome $a + a_3$ proceeds biphasically with fast and slow phases in the transition from State 4 (nomenclature as in ref.9, but without added inorganic phosphate) to the anaerobic State 5 in intact mitochondria from rat liver oxidizing glutamate, as already demonstrated in submitochondrial particles [10] and in intact mitochondria [1,11]. Addition of ATP to anaerobic mitochondria resulted in decrease in the absorbancy at 608 nm minus 630 nm. Subsequent increase in pH from 6.9 to 8.4 caused increase in the absorbancy at 608 nm minus 630 nm and subsequent decrease in pH from 8.4 to 7.0 caused decrease in the absorbancy at 608 nm minus 630 nm. The difference spectrum in the presence and absence of ATP (B minus A in Fig.1(A)) indicates oxidation of cytochrome a having a peak at 608 nm, as shown in Fig.1(B), in good accordance with the result reported by Wikström

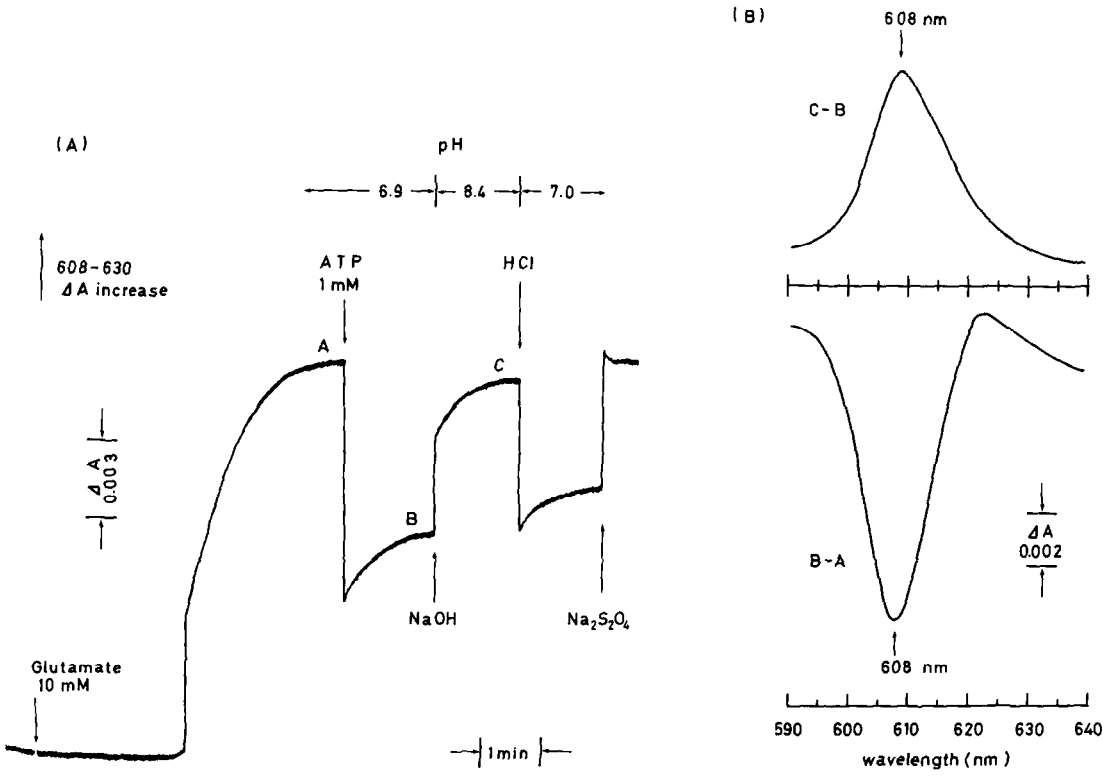

Fig. 1(A) Effects of ATP and pH-transition on the redox state of cytochrome a. State 5 was induced by 10 mM glutamate. 2 mM ATP was added to state 5 mitochondria. The pH transition was obtained by adding about 30 µl of 1 N NaOH and its reversal by adding about 30 µl of 1 N HCl. The rat liver mitochondrial suspension contained 4.6 mg protein/ml.
(B) Absorbance spectra of cytochrome a. Conditions were as for (A) except that 9.2 mg protein/ml of rat liver mitochondria were used. The spectra were obtained with a split-beam spectrophotometer with a slit width of 1.2 nm. Curve B-A, 2 mM ATP was added to the sample cuvette only. Curve C-B, The sample was at pH 8.4 and the reference at pH 7.0.

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[1]. This effect of ATP was abolished by uncoupling agents and by oligomycin as well as by antimycin A (data not shown). It is conceivable, therefore, that oxidation of cytochrome a driven by ATP took place through an energy-linked reversal of electron transfer in the respiratory chain, as described previously [12,1]. The difference spectrum at pH 8.4 minus pH 6.9 (C minus B in Fig.1 (A)) indicates reduction of cytochrome a, as shown in Fig.1(B).

The effect of pH on the redox state of cytochrome a in anaerobic, ATP-treated intact mitochondria from rat liver was reversible between pH 6.5 and 8.5. Essentially the same results as those in Fig.1(A) and (B) were obtained with intact mitochondria from rat heart (data not shown). The pH-dependency of the 608 nm absorbancy is shown in Fig.2. Absorbance changes following addition of 1 N HCl to

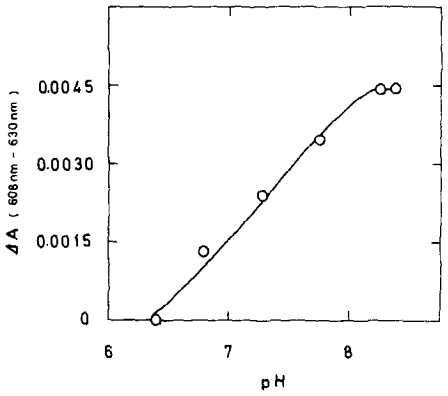

Fig. 2 pH-Dependency of the redox state of cytochrome a. Experimental conditions were as for Fig. 1(A). The pH change was obtained by adding 5 µl of 1 N HCl to 3 ml of anaerobic, ATP-treated mitochondrial suspension (pH 8.4).

anaerobic, ATP-treated mitochondria at pH 8.4 were followed with a two-wavelength spectrophotometer. Fig.2 shows that scarcely any absorbance change was observed above pH 8.0. We also found that the effects of pH on cytochrome b-559 and NAD(P) in anaerobic, glutamate-reduced and ATP-treated intact mitochondria from rat liver were essentially the same as that on cytochrome a (unpublished observation).

The reversible effect of pH on the redox state of cytochrome a was not observed with anaerobic, pentachlorophenol-inhibited and glutamate-reduced intact mitochondria from rat liver or with anaerobic, glutamate-reduced intact mitochondria from rat liver without added ATP or with anaerobic, rotenone-inhibited intact

mitochondria from rat liver without added ATP and substrate. These observations indicate that the reversible effect of pH on cytochrome a was observed only in mitochondria in an energized state, induced by addition of ATP. It seems likely, therefore, that the reversible effect of pH on cytochrome a is essentially different from that on cytochromes b, in which the effect was observed with anaerobic mitochondria, treated with uncoupling agents [13-15]. The present findings appear to favour the chemiosmotic hypothesis, but do not exclude the possibility that OH^--induced reduction of cytochrome a in anaerobic, ATP-treated, intact mitochondria is due to acceleration of a reaction step that involves release of protons, as proposed by Wikström [16] to explain the reversible effect of pH on cytochromes b. However, no evidence has been obtained for direct coupling of proton-dissociation to oxidation of cytochrome a [17]. Moreover, Palmieri and Klingenberg [18] reported that cytochrome a + a_3 is located on the inner side of the inner membrane. Therefore, if the latter explanation is correct, then to explain the present results it must be postulated that an unknown component located outside the inner membrane, which causes release of protons, is involved in OH^--induced reduction of cytochrome a. The effect of pH on cytochrome a in sonicated mitochondrial particles, in which the particle membranes are inside out, will be described in a subsequent paper.

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