Relationships of respiratory chain and ATP-synthetase in energized mitochondria

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The present study reveals that the previously described effect of ATP-synthetase inhibition concomitant with inhibition of respiratory chain functioning may be observed at different absolute values of $\Delta\psi$ on the mitochondrial membrane. This fact points out that the membrane potential is not a unique regulator in coupling of ATP-synthetase and respiratory chain activities. We found, using the double-inhibitor titration technique, that ATP-synthetase inhibition induces proportional inhibition of respiratory chain enzymes and vice versa respiratory chain inhibition induces proportional inhibition of ATP-synthetase. This effect is shown to exist only when osmolarity is close to 150–300 (mosM) (in the physiological range). The coupling effectivity (ADP/O) of mitochondria under these conditions is maximal. Under conditions of high osmolarity (400–600 mosM) the respiratory chain and ATP-synthetase behave as if they were coupled by bulk phase $\Delta\bar{\mu}_{H^+}$, from the kinetic point of view.

ATP-synthetase *Respiratory chain* *Membrane potential*

1. INTRODUCTION

We have previously demonstrated [1,2] that on inhibition of respiration and phosphorylation of rat liver mitochondria in state 3 by malonic acid or sodium cyanide, the membrane potential changes rather insignificantly. These results were recently confirmed in [3]. Authors in [4] observed this effect with submitochondrial particles. Similar effects of the inhibitors of electron transport chain on $\Delta\bar{\mu}_{H^+}$ and ATP synthesis rate were reported with bacterial chromatophores [5,6]. The results of these studies may be interpreted in terms of the existence of interaction between the respiratory chain enzymes and mitochondrial ATP-synthetase [1], which is more localized than that mediated by the bulk phase $\Delta\bar{\mu}_{H^+}$ only.

Some other experimental facts are also interpreted by the authors in terms of the suggestion that the complex of the respiratory chain and ATP-synthetase functions as a 'molecular machine' [7–9]. According to the definition in [10,11], the notion of the complex of the respiratory chain enzymes and ATP-synthetase as a molecular machine involves energy transfer from the respiratory chain to ATP-synthetase via the selected degrees of freedom.

The author in [12] was the first to suggest a model of proton coupling based on structural interaction of the enzymes. Detailed versions of this scheme were presented later.

The 'local chemiosmosis' scheme was proposed in [13,14]. The authors suggest the formation of a 'supercomplex' of the respiratory chain enzymes and ATP-synthetase that ensures direct proton transfer from the respiratory chain to ATP-synthetase. In 1981 an hypothesis was put forward about the existence of certain proteins in *Escherichia coli* responsible for the proton transfer

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along the membrane from the electron transfer chain to ATP-synthetase [15–17].

It was found, using the double-inhibitor titration technique [18], that the localization of free-energy transfer during electron transport phosphorylation by bacterial chromatophores is apparently complete [5,6,19].

A detailed analysis of these works was given in [20]. The author supposed that the local electric field that arises during the charge transfer by the redox enzyme contributes to the driving force for ATP-synthetase localized in the vicinity of the redox enzyme. This point of view, in contrast to the schemes in [13–17] does not require revision of the crucial postulate of the chemiosmotic theory assuming transmembrane charge movement [21,22]. At the same time, it is not necessary to speculate about the existence of special mechanisms of microchemiosmotic coupling; e.g., direct interaction between redox enzymes and ATP-synthetase, or proteins that carry protons along the membrane.

In our work [1,2] we discussed the existence of two regimes of oxidative phosphorylation functioning in mitochondria, as a result of a transmembrane or lateral path of proton movement.

Below we report the data which confirm that under certain conditions the decrease in the number of functioning respiratory chains results in the corresponding decrease in the number of the functioning ATP-synthetase complexes with no $\Delta\psi$ lowering. The increase in osmolarity of the incubation medium is found to abolish this effect.

2. METHODS

Rat liver mitochondria were isolated by differential centrifugation in a medium containing 250 mM sucrose, 5 mM Tris–HCl, 250 μM EDTA, pH 7.8 [23]. Protein in the mitochondrial suspension was assayed by the biuret method with bovine serum albumin as a standard [24].

Mitochondria were incubated in a medium containing 0.2 M sucrose, 10 mM Tris–HCl, 10 mM KH_2PO_4, 20 mM succinate, 5 mM $MgSO_4$, 10 mM KCl, 0.5 mM EDTA, 10 mM glucose, 3.1 units hexokinase, 3.7 units glucose-6-phosphate dehydrogenase, 1 mM $NADP^+$, 40 μM Ap_5A and 2 μM rotenone (pH 7.5).

The respiration rate of the mitochondria was measured polarographically with a Clark electrode. The transmembrane potential was measured by the distribution of tetraphenylphosphonium between the mitochondrial matrix and the incubation medium with a selective electrode [25]. The 'slope' of the electrode retained a Nernst dependence until the concentration of tetraphenylphosphonium in the incubation medium reached 5×10^{-7} M. The internal volume of the mitochondria in media of different osmolarity was taken from [26].

The rate of synthesis of ATP was determined by the reduction of $NADP^+$ to NADPH at 340–370 nm in an Aminco-DW-2A double-beam spectrophotometer. The activity of adenylate kinase was specifically inhibited by Ap_5A [27].

3. RESULTS

As stated above, there have been indications [1,2] that the inhibition of the respiratory chain by malonic acid (fig.1B) induces the inhibition of ATP-synthetase in mitochondria (fig.1A).

Fig.1A,B shows the results of the experiments in which the dependence of the phosphorylation and respiration rates of mitochondria on the concentration of malonic acid was studied at different concentrations of ADP. Addition of malonic acid inhibited both the rate of ATP synthesis (fig.1A) and phosphorylating respiration (fig.1B). These

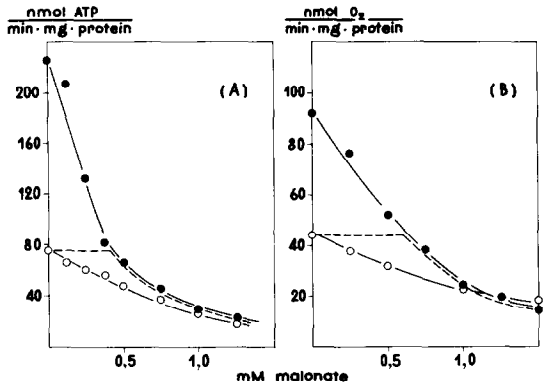

Fig.1. Decrease in the rates of phosphorylation (A) and respiration (B) in rat liver mitochondria in the presence of 500 μM ADP (●) or 5 μM ADP (○) on inhibition of succinate-oxidase activity by malonic acid. For the composition of the incubation medium see section 2. Protein concentration: (A) 0.07 mg/ml; (B) 1.5 mg/ml.

experiments are a modification of the double-inhibitor titration procedure [18,19], where oxidative phosphorylation is limited by addition of low (lower than K_m) concentrations of a substrate rather than by covalent modification of a part of ATP-synthetases with a specific inhibitor [19].

The dashed line in fig.1A,B shows the theoretical inhibition curves [18,19] for the respiratory chain and ATP-synthetase being coupled by bulk phase $\Delta\bar{\mu}_{H^+}$.

Inhibition of the phosphorylating respiration of mitochondria by malonic acid after 30–70% inhibition of respiration by carboxyatractyloside (fig.2A) gave the same results as the use of low concentrations of ADP (fig.1B).

Fig.3 shows the results of the potential measurements on the mitochondrial membrane on addition of low (50 μM) and high (500 μM) concentrations of ADP at different concentrations of malonic acid. The membrane potential and the respiration rate were measured simultaneously. One can see that the relative changes in membrane potential in both cases are not large whereas their absolute values differ greatly. The absolute values of $\Delta\bar{\mu}_{H^+}$ are also significantly different in these experiments. Addition of 15 mM potassium acetate did not affect the results of the experiment.

In the second part of the work it has been demonstrated that the osmolarity of the incubation

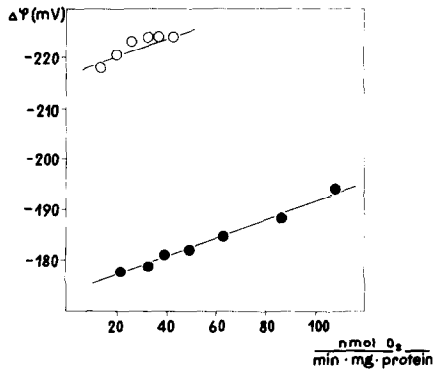

Fig.3. Respiration rates of mitochondria in state 3 and the membrane potential values ($\Delta\psi$) on inhibition of succinate-oxidase activity by malonic acid at different concentrations of ADP. (●) 500 μM ADP; (○) 50 μM ADP. Protein concentration, 1 mg/ml. Concentration of tetraphenylphosphonium, 20 μM.

medium is a physical parameter that controls interaction between the respiratory chain enzymes and ATP-synthetase.

It is seen from fig.2B,4 that under non-physiological conditions, i.e., high osmolarity (400–600 mosM), the system of oxidative phosphorylation undergoes qualitative changes. On the one hand, upon inhibition of respiration by malonic acid, the value of the membrane potential under these conditions considerably changes (fig.4). On the other hand, in the double-inhibitor

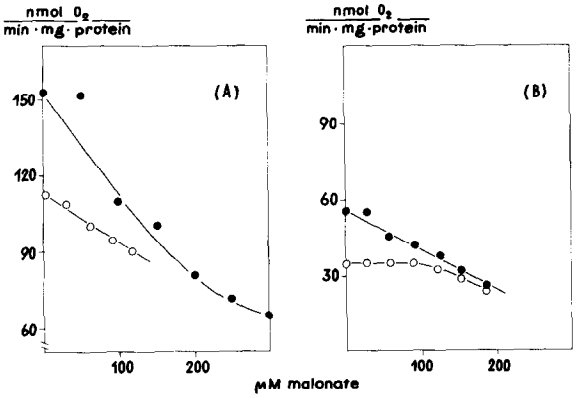

Fig.2. Respiration rate of mitochondria *vs* concentration of malonic acid in media of osmolarity (A) 300 mosM; (B) 600 mosM. (○) In the presence of 2.5×10^{-8} M carboxyatractyloside, (●) without carboxyatractyloside. For the composition of the incubation medium see section 2; 500 μM ADP was added.

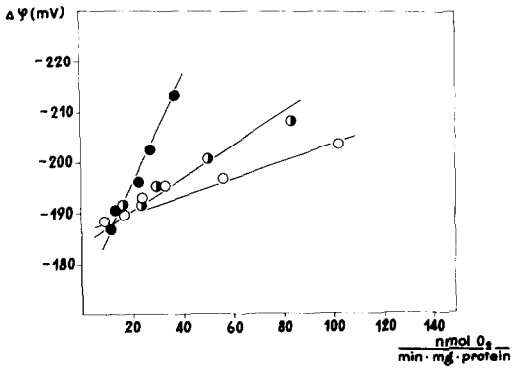

Fig.4. Respiration rates of mitochondria in state 3 and the membrane potential values on inhibition of succinate-oxidase activity by malonic acid at varying osmolarity of the incubation medium. Osmolarity was varied by sucrose. Concentration of ADP, 500 μM. (●) 500 mosM, (◑) 300 mosM, (○) 100 mosM.

titration by malonic acid in the presence of carboxyatractyloside (fig.2B) the respiratory chain and ATP-synthetase behave as if they were coupled by bulk phase $\Delta\bar{\mu}_{H^+}$.

The osmolarity of the medium also affects the ADP:O ratio that characterizes the efficiency of the coupling between the oxidation and phosphorylation under these conditions. If the osmolarity is high, the ADP:O ratio is lower than in iso- and hypotonic media (table 1).

Table 1

ADP:O ratio *vs* osmolarity of incubation medium

Experiment	Osmolarity (mosM)	Temperature (°C)	ADP:O
1	200	21	1.95
	300	21	1.95
	400	21	1.33
2	100	21	1.54
	300	21	1.57
	450	21	1.00
3	100	21	1.52
	300	21	1.39
	600	21	1.10
4	100	21	1.42
	300	21	1.30
	450	21	0.98
5	100	21	1.75
	300	21	1.67
	500	21	0.79
6	300	25	1.39
	500	25	0.78
	500	39	1.31
7	300	24	1.60
	450	24	0.00
	450	35	1.06
8	100	21	1.79
	300	21	1.90
	450	21	1.09
	450	37	1.99
9	300	21	1.82
	400	21	1.53
	450	21	1.10
	300	32	1.80
	400	32	1.88
	450	32	1.33

4. DISCUSSION

It was believed previously that the coupling of the respiratory chain and ATP-synthetase can be controlled by delocalized membrane potential, since this parameter has a threshold value, below which the ATP-synthetase complex cannot function [1]. The results of the experiments presented in fig.1A,B,3 show that the operation of the ATP-synthetase may be regulated by the respiratory chain functioning at different absolute values of membrane potential. This means that the membrane potential is not the only regulator that controls the operation of ATP-synthetase and the respiratory chain enzymes in mitochondria.

On the other hand, double-inhibitor titration indicates (fig.1A,B,2A) that the number of functioning respiratory chains is proportional to the number of the functioning ATP-synthetases.

Our data have shown that osmolarity, a physical parameter of the incubation medium, controls the state of the oxidative phosphorylation system. At values of this parameter close to the physiological level, ATP-synthetase and the respiratory chain behave as a single complex (fig.2A,4). Under these conditions the efficiency of coupling (ADP:O) is maximal (table 1).

Under the condition of high osmolarity (400–600 mosM) the system of oxidative phosphorylation in mitochondria appears to have another regime of functioning: from the kinetic point of view the respiratory chain and ATP-synthetase behave as enzyme complexes, coupled by bulk phase $\Delta\bar{\mu}_{H^+}$.

A similar result was found on addition of polyvinylpyrrolidone to mitochondria counterbalancing osmotic pressure of proteins in the intermembrane space of mitochondria (V.P. Skulachev, personal communication) [13].

The results of the experiments described above may be explained in terms of direct interaction of the respiratory enzymes and ATP-synthetase, as a result of which the inhibition of the ATP-synthetase complex induces synchronous inhibition of the respiratory chain enzymes and, vice versa, inhibition of the respiratory chain synchronously inhibits ATP-synthetase.

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