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Flux-yield dependence of oxidative phosphorylation at constant $\Delta\tilde{\mu}H^+$

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By using an NADH-regenerating system, different steady states were obtained in which the electron flux through each respiratory unit was modulated. Under these conditions it was observed that, at constant $\Delta\tilde{\mu}H^+$, the ATP/O value decreased with increasing electron flux. This flux-yield dependence can be regarded as evidence in support of indirect coupling at the proton pump level.

Oxidative phosphorylation; ATP/O stoichiometry; Mitochondria; (Yeast)

1. INTRODUCTION

Knowledge of mechanistic stoichiometries in coupling between respiration and phosphorylation catalysed by mitochondria is of considerable interest for the understanding of the energetics of cells. As a matter of fact, conflicting results in the determination of these stoichiometries have been the object of a great deal of discussion concerning isolated mammalian mitochondria [1-3]. The problem in coming to an agreement appears to be partially one of technical difficulties (review [4]). However, some experiments have suggested a change in stoichiometry of proton pumping by the electron transport chain [5-9] or by the ATPase [6]. If one considers such a possibility, ATP/$2e^-$ stoichiometries, measured at the individual coupling sites, can vary according to the experimental conditions used for determination.

In this paper, we show that the value of ATP/O increases when respiratory flux slows down in response to a limitation of substrate supply. Such a flux-dependent change, without any relation with a variation of $\Delta\tilde{\mu}H^+$, could be due to a variability in the degree of coupling of proton pumps implicated.

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2. MATERIALS AND METHODS

2.1. *Preparation of mitochondria*

Cells of diploid wild-strain *Saccharomyces cerevisiae* (yeast foam) were grown aerobically at 28°C in a complete medium (pH 4.5) with 2% lactate as carbon source. The cells were harvested in logarithmic growth phase and mitochondria were isolated from protoplasts as described in [10]. Protein concentration was measured by the biuret method using bovine serum albumin as a standard.

2.2. *Determination of ATP/O*

Mitochondrial oxygen consumption was measured at 27°C in a 1.5-ml thermostatically controlled chamber equipped with a Clark oxygen electrode (Gibson) connected to a microcomputer giving an on-line display of rate values. Basal medium was as follows: 0.65 M mannitol, 0.36 mM EGTA, 3 mM Tris-P_i, 10 mM Tris-maleate (pH 6.7), 10 μM RbCl and 0.01 μg valinomycin/ml. Substrate supply was either a saturating concentration of different substrates or a NADH regenerating system: 4 mM glucose 6-phosphate, 2 mM NAD^+ and various amounts of glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The rate of ATP synthesis was initiated by addition of 1 mM ADP; after acid extraction, ATP was measured by 3 different methods: (i) $^{32}P_i$ incorporation in adenine nucleotides as previously described in [11]; (ii) glucose 6-phosphate formation in the presence of non-limiting amounts of hexokinase, 1 mM $MgCl_2$ and 10 mM glucose, or (iii) by disappearance of P_i in the incubation medium. P_i concentration was determined according to [12].

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2.3. *Measurement of ΔpH and Δψ*

Matrix space was determined by using [^{3}H]water and inner membrane impermeable [^{14}C]sucrose, $\Delta\psi$ and ΔpH by distribution of ^{86}Rb (in the presence of valinomycin) and [^{3}H]acetate, respectively [13]. Routinely, after equilibration (3 min), mitochondria were separated from the medium by rapid centrifugation (30 s) through a silicone oil layer (silicone AR200 fluid).

2.4. *Determination of NAD$^+$ and NADH*

NAD$^+$ and NADH concentrations were determined by the technique described in [14].

3. RESULTS AND DISCUSSION

Mitochondria isolated from *S. cerevisiae* in exponential phase of growth present 3 main characteristics: (i) lack of phosphorylation site corresponding to coupling site 1 of animal mitochondria [15–17]; (ii) ability to oxidize exogenous NADH by a NADH dehydrogenase located towards the outer surface of the inner membrane [18]; (iii) the ability to oxidize lactate by using directly the third site span [15]. Our first aim was to determine ATP/O stoichiometries at sites 2 + 3 and 3 by applying an experimental procedure which minimized the effect of endogenous uncoupler.

Tsou and Van Dam [19] have shown that low concentration of uncoupler depresses phosphorylation rate at every rate of respiration but has little effect on the slope of the linear relationship between respiratory and phosphorylation rates when these rates vary with respiratory chain inhibitors. Consequently, the effect of endogenous uncoupler can be largely avoided by determining the ATP/O or ATP/2e$^-$ stoichiometry from the slope of this relationship. Such a property has been confirmed and used in previous studies with mammalian mitochondria [1,3]. This approach appears to be particularly suitable for measuring the ATP/O ratio in yeast mitochondria where the state 4 respiration is always higher than in mammalian mitochondria. Fig.1A shows an antimycin titration of phosphorylation and respiratory rates with external NADH as substrate. Each of the techniques used to measure ATP could lead to misestimations of phosphorylation rate, i.e., either an underestimation due to ATPase contaminating activity (^{32}P incorporation or P_i disappearance methods) or an overestimation mainly due to adenylate kinase activity (glucose 6-phosphate method). However, whatever the method used for ATP estimation, the

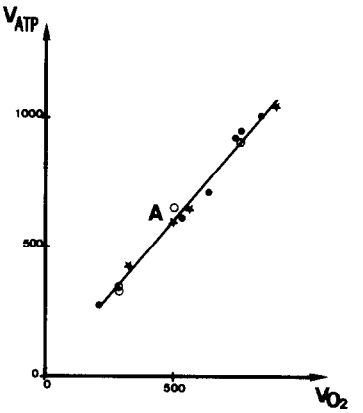

Fig.1. Measurement of ATP/O at sites 2 + 3 and 3 by respiratory chain inhibitor titrations. Mitochondria (0.6 mg protein) were suspended in 1.5 ml respiratory medium supplemented with (A) 2 mM NADH, (B) 5 mM succinate and (C) 3 mM TMPD + 4 mM ascorbate. In (A), 3 different methods were used for ATP measurements (see section 2): either $^{32}P_i$ incorporation into adenine nucleotides (★) or glucose 6-phosphate formation (●) or P_i disappearance (○); inhibitors used were antimycin in (A,B) or cyanide in (C). V_{ATP} is given as nmol ATP · min^{-1} · mg^{-1} protein and V_{O_2} as ngatom O · min^{-1} · mg^{-1} protein.

experimental points were distributed on the same straight line, indicating that neither contaminating ATPase activity nor adenylate kinase activity changed significantly ATP synthesis rate estimations (fig.1A). By using the experimental procedure presented in fig.1A, with antimycin or cyanide as inhibitors, ATP/O ratio was determined with different respiratory substrates. It appeared that ATP/O was about 1.5 with succinate (see fig.1B) or ethanol (not shown) or 1 with substrates oxidized at site 3 as TMPD + ascorbate (fig.1C) or lactate (not shown). These results are in agreement with those obtained recently in mammalian mitochondria [3]: 1.5 and 1 at sites 2 + 3 and 3, respectively. However, with substrates considered to donate their electrons to site 2 the external side of the membrane, such as NADH (see fig.1A) or glycerol phosphate (not shown), ATP/O quotient is about 1.25. Thus, ATP/O values seem to depend on location of the dehydrogenase either on the outer or inner faces of the internal membrane. This difference seems linked to the site 2 span where ATP/2e$^-$ with NADH as substrate is only half of that with ethanol or succinate. From a physiological point of view, it is interesting to note

that phosphorylation with NADH is only slightly higher than that with lactate which donates its electrons directly to the site 3 span.

Since yeast mitochondria possess a NADH dehydrogenase located towards the outer surface of the inner membrane, it is easy to limit the respiratory rate by NADH supply. For such a purpose, mitochondria were incubated in a medium containing a NADH regenerating system, as described in section 2. At various steady states, obtained by different amounts of glucose-6-phosphate dehydrogenase, respiratory and phosphorylation rates were determined. As shown in fig.2A, the ATP/O ratio decreases as a function of respiratory rate, from 1.8 for 120 ngatom $O \cdot min^{-1} \cdot mg^{-1}$ protein to 0.95 for 400 ngatom $O \cdot min^{-1} \cdot mg^{-1}$ protein, and then remains constant. The ATP/O value obtained in this way, at high respiratory rate, is lower than that measured by antimycin titration when NADH concentration is saturating (cf. figs 1A and 2A). However, it should be noted that the experimental conditions are different. For instance, as shown in fig.2B, the NADH/NAD^+ value is always below 0.7 when using the regenerating system; when 2 mM NADH is used as substrate (fig.1A) it is calculated that at the end of the experiment, the NADH/NAD^+ value is still higher than 5.

A change in ATP/O stoichiometry indicates

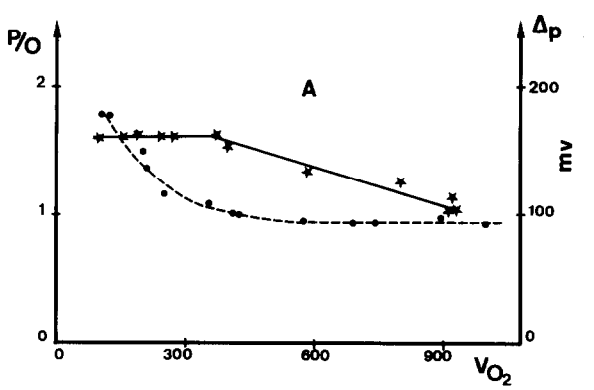

Fig.2. Dependence of ATP/O, $\Delta\tilde{\mu}H^+$ and NADH/NAD^+ ratio on respiratory rate. Mitochondria were suspended in 1.5 ml respiratory medium containing a NADH regenerating system (see section 2) with various amounts of glucose-6-phosphate dehydrogenase. For a given enzyme concentration, ATP/O (●), $\Delta\tilde{\mu}H^+$ (★) (A), and NADH/NAD^+ ratio (B) were determined at steady state. ATP formation was estimated by $^{32}P_i$ incorporation into adenine nucleotides (see section 2).

either a modification in the stoichiometry of at least one of the proton pumps involved (i.e., respiratory chain or ATP synthase), or a variation in proton leak. As shown in fig.2A, $\Delta\tilde{\mu}H^+$ measured during phosphorylation is constant when respiratory rate increases up to 400 ngatom $O \cdot min^{-1} \cdot mg^{-1}$ protein. Above this value, $\Delta\tilde{\mu}H^+$ decreases slowly when respiratory rate rises. The main observation is that ATP/O value decreases in a flux-dependent manner at constant $\Delta\tilde{\mu}H^+$. Therefore, this change in ATP/O is not a consequence of proton leak modification but must be due to alteration of either the $H^+/2e^-$ stoichiometry of the respiratory chain or the H^+/ATP stoichiometry of ATP synthase, or both. A change in stoichiometry of proton pumping by the respiratory chain of rat liver mitochondria has been reported [8,9]. However, in the present experiments, the $H^+/2e^-$ stoichiometry decreases as a function of increasing $\Delta\psi$. These results indicate that the yield of oxidative phosphorylation depends both on $\Delta\tilde{\mu}H^+$ [8,9] and on flux (this paper) values.

Limitation of respiratory activity by using a NADH regenerating system is a fundamentally different approach from that using inhibitor titration. In fact, in the first method, the electron flux through each respiratory unit is modulated, even when, in the second procedure, the number of functional units is changed. Consequently, the first method is most suitable for investigating relationships between the flux and the reaction yield.

The flux-yield dependence which is a general feature of the behaviour of a working engine can be taken as evidence in favour of indirect coupling at the pump level.

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