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# Activation by ATP of a proton-conducting pathway in yeast mitochondria

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The growth of *Saccharomyces cerevisiae* cells under aerobic conditions, in the presence of an energy-rich source, leads to production of an excess of NAD(P)H. Since the redox balance must be maintained, it has been postulated that NAD(P)H reoxidation is accelerated by the activation of energy-dissipating reactions, which would, in turn, explain the low growth efficiencies observed. It has been demonstrated already in *S. cerevisiae* cultures that these putative energy-dissipating reactions are stimulated both by oxygen and high cytosolic ATP levels. In this paper, we show that ATP induces a proton-permeability pathway in mitochondria at concentrations which are within the physiological range, as revealed both from the ATP stimulation of respiration and from the induction of **In**-dependent swelling. We also demonstrate that phosphate acts as a competitive inhibitor of the nucleotide, and since activation is observed even in the presence of atractylate, we postulate that the ATP-binding site is located in the outer face of the mitochondrial inner membrane.

Over the past decade, it has become clear that when yeasts are grown under aerobic conditions, the growth yield/mol ATP produced is not constant and depends greatly on the nature of the substrate utilized [1–5]. If cells are growing on a single carbon source, this substrate must serve two functions; as a source of carbon skeletons for biosynthesis and as a source of reducing equivalents both for energy production and for biosynthesis. Therefore, depending on the degree of reduction of the substrate, the culture may be energy-limited (if the substrate is too oxidized) or carbon-limited (carbon supply is limiting growth while energy generation occurs in excess) [2, 3]. In fact, it has been demonstrated that the more oxidized the substrate, the higher the growth efficiency [5]. Since growth in glucose, ethanol and glycerol, amongst others, is carbon-limited and the redox balance must be maintained, it has been proposed that excess reducing equivalents are eliminated in oxidative processes which are non-energy conserving [6]. The relative importance of these energy-dissipating reactions becomes apparent when the theoretical ATP requirements for biomass formation are calculated and compared with the expected ATP yield of substrate oxidation; then it is observed that they differ by a factor of two [4]. One of the reasons for this discrepancy may be the difficulty in assessing ATP generation rates, since a basic parameter, the P:O ratio, is not known with sufficient accuracy [3, 7] and, in fact, a number of studies have revealed that the *in-vivo* P:O ratios must be significantly lower than the mechanistic values (review in [4]). This may reflect the existence of strategies for the adjustment of the rates of ATP production and of energy dissipation. One mechanism which exemplifies the regulation of the ATP production rate is the deletion of oxidative phos-

phorylation sites by induction of NAD(P)H dehydrogenases which do not translocate protons [3, 8, 9]. With respect to energy-dissipating reactions, three putative mechanisms have been proposed; wastage of ATP by an ATPase, increased membrane proton conductance and the presence of futile cycles [3, 10–12].

This low efficiency, observed at the cellular level, is also encountered with isolated mitochondria as revealed both by low P:O ratios and a poor ADP stimulation of respiration [13–16]. Low P:O ratios are partially explained by the presence of two rotenone-insensitive dehydrogenases, one for NADH and another for NADPH, both accessible from the cytosolic side of the mitochondrial membrane and which do not translocate protons [8, 9]. In fact, the presence of these dehydrogenases may indicate that cytosolic overproduction of reduced coenzymes does occur and that mitochondrial respiration helps in the maintenance of the redox balance [6]. Since no explanation for the endogenous loose coupling has been found, the existence of other energy-dissipating reactions has remained hypothetical.

The results presented here may explain the low efficiency of oxidative phosphorylation in yeast, since we demonstrate that these mitochondria possess a proton-conductance pathway activated by ATP and inhibited by phosphate and that when active, it uncouples respiration from ATP synthesis. This system is analogous to the heat-generating system of brown adipose tissue mitochondria; fast respiration is due to dissipation of the proton electrochemical-potential gradient through the uncoupling protein (UCP) [17, 18].

## MATERIALS AND METHODS

### Strains, media and isolation of mitochondria

Preliminary experiments were performed with two diploid strains of *S. cerevisiae* which are convenient for recombinant

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Abbreviations. UCP, uncoupling protein; YUP, yeast uncoupling pathway; UR, uncoupling ratio.

expression of foreign proteins. Strains W303 (aa,  $ade^{2-}$ ,  $his^{3-}$ ,  $leu^{2-}$ ,  $ura^{3-}$ ,  $trp^{1-}$ ) and LB6 (aa,  $leu^{2-}$ ) were used and results were identical. The rest of the work has been carried out using the strain W303. Yeasts were grown aerobically at 28°C in a minimal medium which contained 6.7% yeast nitrogen base without amino acids (Difco), 1% sodium phosphate, 1.3% ammonium sulfate, appropriate amino acid supplements and 2% lactate plus 0.1% glucose as carbon source, pH 4.5. For respiration experiments, yeast were grown in a complete medium which contained 1% yeast extract (Difco), 2% bactopectone (Difco) and 2% lactate as carbon source, pH 4.5. Yeast cells were harvested in logarithmic-growth phase, protoplasts prepared by enzymatic digestion with cytohelicase (1BF Biotechnics), and mitochondria prepared by differential centrifugation after homogenization of protoplasts [19]. Mitochondrial buffers contained 0.6 M mannitol, 2 mM EGTA, 0.5 mM sodium phosphate, 2% albumin and 10 mM Tris/maleate, pH 6.8. Mitochondria from rat liver and from brown adipose tissue of cold-adapted hamsters were prepared as previously described [20].

### Determination of proton permeabilities

Rates of proton permeation through the mitochondrial inner membrane were determined at 18°C, from the rate of light-scattering decrease during osmotic swelling in potassium acetate plus valinomycin [21]. Incubation medium contained 0.1 M potassium acetate (rat liver or brown adipose tissue mitochondria) or 0.2 M potassium acetate (yeast mitochondria), 2 mM  $MgCl_2$ , 2 mg/ml albumin, 10 pM atractylate, 12.5 pg/ml oligomycin, 0.5 pg/ml antimycin A, 10 mM Tris/maleate, pH 6.8. The protein concentration was 0.5 mg/ml for yeast mitochondria and 0.3 mg/ml for brown adipose tissue and rat liver mitochondria. The basal proton permeability was calculated from the rate of swelling after addition of 1.3 pM valinomycin but prior to the addition of the nucleotide.

### Oxygen-electrode measurements

Yeast mitochondria (0.5 mg/ml) were incubated at 18°C in a Hansa-Tech oxygen electrode chamber (Hansa-Tech) in medium containing 3 mM  $\alpha$ -glycerophosphate as substrate, 0.65 M mannitol, 2 mM  $MgCl_2$ , 0.5 mM EGTA, 2 mg/ml bovine serum albumin and 10 mM Tris/maleate, pH 6.8.

## RESULTS AND DISCUSSION

Under conditions of uncoupled growth, defined as a situation in which the growth yield is lower than expected on the basis of the theoretical ATP yield [2, 3, 22], the existence of energy-dissipating mechanisms has been postulated [3, 10–12]. Since the observed PrO ratios are lower than expected [13–16], it would be possible that the energy-wasting mechanisms involve dissipation of the proton electrochemical-potential gradient. If this were the case, analysis of the proton permeability of the mitochondrial inner membrane could provide an insight into possible pathways for proton re-entry into the matrix. A simple technique which allows monitoring of mitochondrial proton permeability is to follow the rate of osmotic swelling in mitochondria suspended in potassium acetate in the presence of valinomycin; swelling will be limited by the rate at which protons entering with acetate can leave

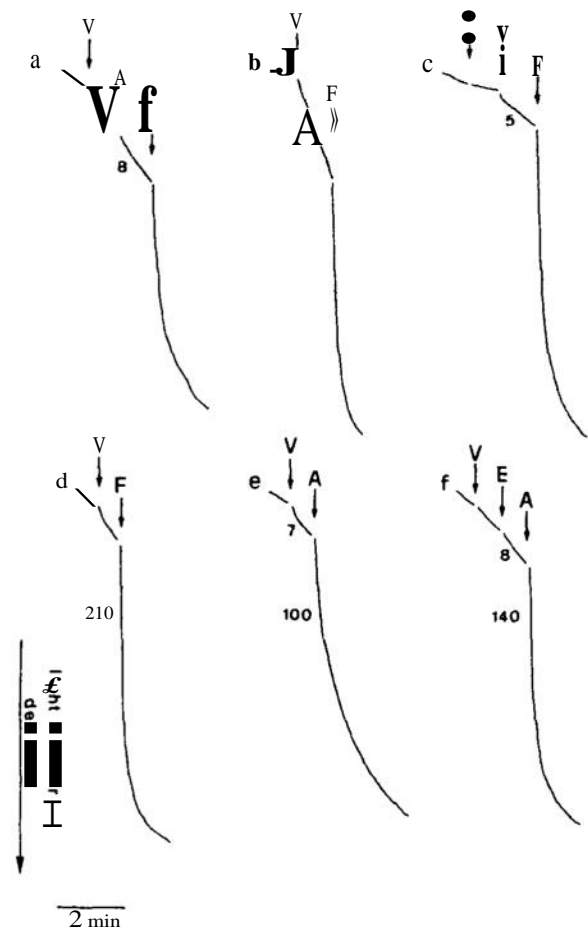
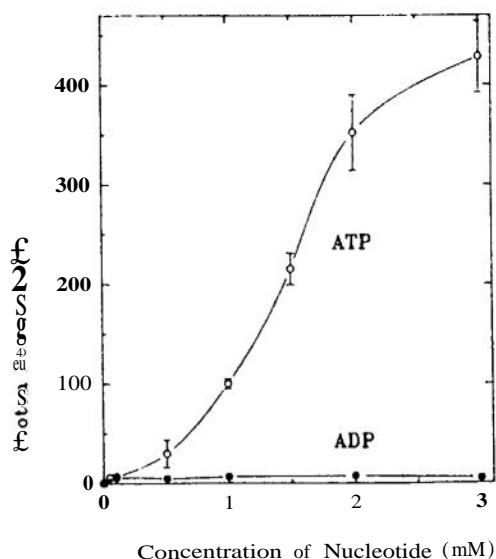


Fig. 1. Effect of ATP on the proton permeability of mitochondria from different sources. (a) Mitochondria from rat liver; effect of ATP and induction of proton permeability by carbonyl cyanide p-trifluoromethoxyphenylhydrazone. (b) Mitochondria from brown adipose tissue, swelling in the absence of nucleotides; (c) mitochondria from brown adipose tissue, swelling in the presence of 1 mM ATP plus carbonyl cyanide p-trifluoromethoxyphenylhydrazone. (d) Yeast mitochondria, swelling induced by carbonyl cyanide p-trifluoromethoxyphenylhydrazone; (e) yeast mitochondria, ATP effect; (O) influence of EGTA on swelling of yeast mitochondria induced by ATP. Additions: V, 1.3 pM valinomycin; A, 1 mM ATP; F, 3 pM carbonyl cyanide p-trifluoromethoxyphenylhydrazone; E, 1 mM EGTA. Figures represent swelling rates in arbitrary units.

the matrix to maintain the charge balance [21]. Fig. 1 presents a comparison of the native proton permeability of mitochondria from three different sources, rat liver, hamster brown adipose tissue and *S. cerevisiae*, showing that while hamster brown adipose tissue mitochondria are permeable to protons (Fig. 1b), the other two will only swell if an uncoupler (in our case, carbonyl cyanide p-trifluoromethoxyphenylhydrazone) is present (Fig. 1a and d). The enhanced permeability to protons of hamster brown adipose tissue mitochondria is due to UCP, a proton transporter which is inhibited by purine nucleotides and activated by fatty acids [17, 18].

Two main observations point to a role of ATP in the activation of the putative energy-dissipating mechanisms of yeast mitochondria. First, it has been shown in cultures of *S. cerevisiae* that energy-dissipating reactions are stimulated both by oxygen and high cytosolic ATP levels [10]. Second, in isolated mitochondria when ATP is produced by phosphorylation of ADP, respiratory control is partially lost [13], an effect

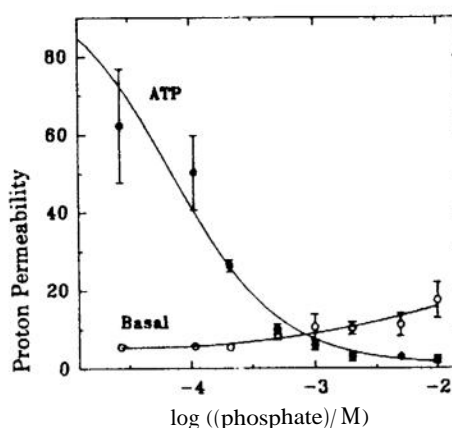


**Fig. 2. Effects of ATP (O) and ADP (●) on the proton permeability of *S. cerevisiae* mitochondria.** Permeabilities were determined from the rate of swelling of mitochondria in potassium acetate plus valinomycin. Swelling rates are given in arbitrary units, taking the permeability induced by addition of 1 mM ATP as 100. Results represent the mean  $\pm$  S. E. M. of 3–5 independent experiments. Curves have been fitted to experimental data using a non-linear least-squares method.

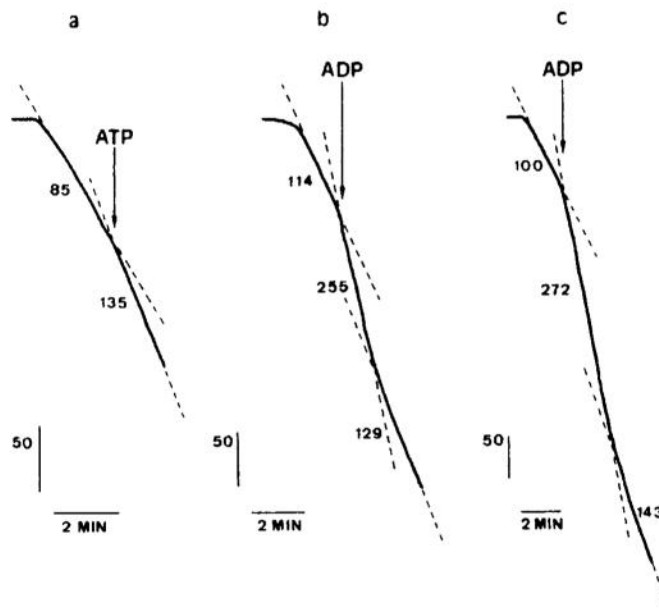
which is apparent whenever O<sub>2</sub>-electrode traces are shown [8, 23, 24]. Thus, in Fig. 1, the effects of ATP on the proton permeability of the three types of mitochondria were investigated. ATP had no effect on rat liver (Fig. 1a); as expected it inhibits swelling in hamster brown adipose tissue by inhibiting UCP (Fig. 1c) [17, 18] and, interestingly, it greatly increases the proton permeability of yeast mitochondria (Fig. 1e). Activation in yeast mitochondria of a K<sup>+</sup>/H<sup>+</sup> antiporter can be ruled out since, in the absence of valinomycin, addition of ATP does not trigger swelling. Induction of proton permeability by ATP does not depend on its translocation into the matrix, since the experiments were performed in the presence of atractylate and, in control experiments the measurement of [<sup>3</sup>H]-ATP binding and uptake was checked to ensure that, indeed, the import of nucleotides was blocked (data not shown). A possible chelating effect of nucleotides can be ruled out since 1 mM EGTA does not induce swelling (Fig. 1f). Since artifacts appear when there is depletion of Mg<sup>2+</sup>, probably due to activation of the inner-membrane anion channel [25], our experiments were always performed with 2 mM MgCl<sub>2</sub>.

Fig. 2 shows the concentration range in which ATP induces the leak. Results reveal that the pathway activation follows sigmoidal kinetics, suggesting positive cooperativity, but what is more important is that the ATP concentrations required are within the levels found in the cytosol. In contrast, ADP shows no significant effect on proton permeability. Since phosphate has been pointed out as a regulator of state-4 respiration in yeast, acting as a 'coupling factor' [23], we studied the effect of phosphate on swelling, induced by 1 mM ATP (Fig. 3), and observed that low concentrations of phosphate inhibited the ATP-induced proton permeability with a *K<sub>t</sub>* around 60 pM.

In order to reproduce the *in-vivo* situation as closely as possible, it is essential to investigate the effects of ATP under respiring conditions. Fig. 4a shows that addition of 1 mM ATP in the presence of atractylate does cause an increase in



**Fig. 3. Inhibition by phosphate of the ATP-stimulated mitochondrial proton permeability.** Permeabilities were determined in incubations supplemented with concentrations of phosphate ranging from 20 pM to 10 mM. Swelling rates were determined, first after addition of valinomycin (basal permeability), and second once 1 mM ATP had been added. Proton permeabilities are given in arbitrary units, taking the permeability induced by 1 mM ATP in the absence of phosphate as 100. Results are the mean  $\pm$  S. E. M. of 3–5 independent experiments.



**Fig. 4. Effect of ATP on the respiration rate of yeast mitochondria.** (a) Effect of 1 mM ATP on state-4 respiration in the presence of 10 pM atractylate, 12.5 pg/ml oligomycin and 0.5 mM sodium phosphate. (b) Stimulation of respiration by 0.1 mM ADP, and (c) 0.3 mM ADP; both experiments performed in the presence of 1 mM sodium phosphate and in the absence of atractylate and oligomycin. Figures give respiratory rates in nmol O<sub>2</sub> · min<sup>-1</sup> · mg protein<sup>-1</sup>. Vertical bars represent an oxygen content of 50 nmol O<sub>2</sub>/ml. For (a) UR = 1.60, for (b) UR = 1.13 and for (c) UR = 1.43.

the rate of respiration; we have illustrated this uncoupling effect by calculating the ratio between the respiratory rates obtained after and prior to ATP addition, defining this quotient as the 'uncoupling ratio' (UR), which in this case is 1.60. When ATP is produced by phosphorylation of ADP, it can be seen that the rate of respiration obtained when mitochondria return to state 4 depends on the amount of ATP produced, being higher when high concentrations of ADP are phos-

phorylated (Fig. 4b and c). For respiration experiments, W303 yeast cells were grown either in minimal medium or in complete medium (see Materials and Methods); under these conditions, mitochondria showed ATP-induced uncoupling (UR for 0.3 mM ATP was  $1.58 \pm 0.07$  in minimal medium and  $1.41 \pm 0.06$  in complete medium,  $n = 4$ ), but respiratory control ratios (quotient between the state-3 rate and first state-4 rates) were always lower in cells grown in minimal media (for 0.3 mM ADP the ratio was  $1.89 \pm 0.06$  in minimal medium compared to  $2.58 \pm 0.08$  in complete medium). For Fig. 4, we have chosen oxygen-electrode traces of cells grown in complete medium since state 3–state 4 transitions are more pronounced and therefore illustrate more clearly the ATP effects.

It should be noted that, in these experiments, the phosphate concentration was 1 mM, therefore well above the  $K_i$  obtained in swelling experiments (Fig. 3). This discrepancy could reflect the difference in the magnitude of the proton electrochemical-potential gradient, which is around 20 mV during passive swelling and close to 200 mV during respiration, thus as the driving force increases, the concentration of phosphate required to inhibit transport also increases. This situation is also found in brown fat mitochondria, where proton-dependent swelling is inhibited by 10 pM ATP, while during respiration the concentration required to inhibit UCP proton conductance is close to 1 mM [17, 18].

Our hypothesis proposes the existence of a site in the cytosolic face of the inner mitochondrial membrane where ATP binds to induce a proton-conducting pathway (yeast uncoupling pathway; YUP); YUP activity is the result of a balance between the opposing effects of ATP and  $P_i$ , with the overall result of a reduction in the coupling efficiency. The high affinity for phosphate implies that mitochondria should remain coupled if ATP levels are low; however, if ATP levels rise, the phosphate concentration will drop and the YUP activity should increase. This type of regulation would ensure that cells do not become ATP depleted since, if ATP levels fall, the pathway will be inhibited and the phosphorylation efficiency will be recovered.

Our proposal is consistent with bioenergetic data published previously which demonstrated, in isolated mitochondria, a variable ATP:O stoichiometry [15, 26]. This variable stoichiometry has been interpreted as being due to a loose coupling between electron transfer and  $H^+$  extrusion in the respiratory chain [15]. Since our hypothesis postulates that the higher the ATP level the more active the uncoupling pathway, the decrease in the P:O ratio would reflect an increased proton leak. It is important that these observations made with isolated mitochondria offer an explanation for the observations made in cultures: theoretical calculations have revealed that, in *S. cerevisiae*, energy demands for growth and maintenance account for about 30–40% of the total ATP produced theoretically from the amount of substrate respired and the rest is used up in unknown processes [27]. As discussed earlier, aerobic growth on a single carbon source may lead to an imbalance between the rate of NAD(P)H production and its consumption; in order to maintain the redox balance, cells will re-oxidize the excess NAD(P)H by activating energy-dissipating reactions. Two strategies have been described; first, induction of two rotenone-insensitive dehydrogenases [8, 9] and, according to our hypothesis, the activation of YUP. As discussed earlier, it is interesting that an inverse correlation between energetic efficiency and the cytosolic ATP concentration in *S. cerevisiae* has already been demonstrated [10].

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