Deficiency of Uncoupler-stimulated Adenosine Triphosphatase Activity in Yeast Mitochondria

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Oligomycin-sensitive ATPase activity was studied in isolated yeast mitochondria. The protonophore CCCP, at a concentration which completely inhibited ATP synthesis, induced only a low rate of hydrolysis of externally added ATP, and the extent of hydrolysis was dependent upon phosphate (P_i) concentration. CCCP promoted hydrolysis of intramitochondrial ATP. However, hydrolysis of externally added ATP was total in a medium containing potassium phosphate plus valinomycin. Without ionophores, ATPase activity was only observed at high external pH or with detergent-treated mitochondria. Under state 4 conditions, external ATP had access to the catalytic nucleotide site of ATPase as shown by ^32P–ATP exchange experiments. These results are discussed in terms of a limitation of the translocase-mediated ATP/ADP exchange in uncoupled mitochondria.

INTRODUCTION

Uncouplers stimulate the respiration of isolated yeast mitochondria and inhibit some energy linked reactions (Ohnishi et al., 1967; Kováč et al., 1972). However, the ATPase activity, assayed with added ATP, is only observed at alkaline pH; uncouplers do not stimulate ATPase activity at neutral pH (Kováč et al., 1968; Somlo, 1968). These results clearly indicate that a collapse of ΔpH + by uncoupler is not sufficient to unmask ATPase activity. Similar observations have been reported with bacteria (Ferguson et al., 1976), and with plant (Jung & Hanson, 1975) and tumour cell mitochondria (Kolarov et al., 1973; Pedersen & Morris, 1974; Hayashi et al., 1980a,b; Knowles, 1982).

We report here the results of a study to determine whether the ATP-synthase works in a reversible manner, as part of our continuing investigations of the control of the oxidative phosphorylation system in yeast mitochondria (Rigoulet et al., 1983; Jean-Bart et al., 1984).

METHODS

The diploid Saccharomyces cerevisiae strain Foam was grown aerobically with lactate as carbon source; mitochondria were prepared from protoplasts, as described previously (Guerin et al., 1979). Protein concentration was measured by the biuret method using bovine serum albumin as a standard.

ATP synthesis and hydrolysis were assayed at 20 °C in the following basal medium: 10 mM-Tris/maleate, 0·65 M-mannitol, 0·3% (w/v) bovine serum albumin, 0·36 mM-EGTA, pH 6·7, containing 0·15 mg mitochondrial protein ml⁻¹. The particular conditions for synthesis and hydrolysis are given in the legends to the Figures and Table. At defined times, 0·25 ml of the suspension was pipetted into vials containing 0·17 ml cold 0·75 M-TCA and was immediately centrifuged. TCA was removed from the supernatant by extraction with diethyl ether. ATP.

Abbreviations: AdN: adenine nucleotide; P_i: inorganic phosphate; CCCP: carbonyl cyanide m-chlorophenylhydrazone; F: hydrosoluble factor of the mitochondrial ATP-synthase; ΔpH +: transmembrane electrochemical gradient for protons; ΔpH: transmembrane pH gradient; Δψ: transmembrane electrical potential.
ADP and AMP were assayed by the bioluminescence method (Lundin & Baltcheffsky, 1978) using a luminometer 1250 (LKB-Wallac). For the ATP-\(^{32}\)P exchange, mitochondria were suspended in basal medium containing 0.6% ethanol and 4 mM-Tris/phosphate. ATP synthesis was started by addition of 400 nmol ADP. When ATP synthesis was over (after 10 min incubation), the exchange experiment was started by \(^{32}\)P addition, such that the total external phosphate concentration was not significantly increased; exchange was stopped at defined times by addition of TCA (see above). After centrifugation, 0.4 ml of the supernatant was pipetted into vials containing 0.5 ml Norit suspension (100 mg ml\(^{-1}\)). The suspension was thoroughly mixed, filtered through a paper disc (Whatman no. 1), then washed with 0.2 M-potassium phosphate and finally washed with distilled water. Filters were dried and their radioactivity was counted. \(^{32}\)P was purchased from CEA-Saclay (France).

RESULTS

Effect of CCCP on ATP synthesis and hydrolysis. Initial experiments were done to compare the effect of the uncoupler CCCP on the synthesis and hydrolysis of ATP under similar experimental conditions. Fig. 1\((a)\) shows that 5 \(\mu\)M-CCCP largely inhibited ATP synthesis. Without uncoupler, maximal ATP synthesis was achieved 5 min after ADP addition. The effect of CCCP added 10 min after ADP depended on the initial concentration of external Pi. For 0.2 mM-Pi, CCCP induced only a very slow hydrolysis of ATP (Fig. 1\((a)\)). For 4 mM-Pi, about 40% of the ATP was rapidly hydrolysed and then no further hydrolysis was observed (Fig. 1\((b)\)). Mersalyl, an inhibitor of the Pi-carrier, fully inhibited the CCCP-induced ATPase activity when added just before the uncoupler (Fig. 1\((b)\)).

The sum of the amounts of ATP and ADP was constant during the course of the experiments, indicating that the increase in ATP was due to added ADP phosphorylation and vice versa (Fig. 1\((b)\)). The ATPase activity was inhibited by oligomycin (Fig. 1\((a)\)) and only slowly stimulated by Mg\(^{2+}\), suggesting a low contamination of the mitochondrial preparation either by non-mitochondrial fractions or by disrupted mitochondria (not shown). This low Mg\(^{2+}\) stimulation appeared to be largely oligomycin-insensitive.

Effect of CCCP on the endogenous pool of adenine nucleotides. Phosphorylation and hydrolysis of the internal AdN pool was studied without ADP addition (Table 1). In the absence of added respiratory substrate, approximately 60% of the internal AdN pool was AMP. However, this value varied and probably depended on the extent to which the mitochondria were depleted of internal respiratory substrate. Ethanol addition induced a large increase in ATP and a decrease in AMP; ADP content increased slightly. CCCP induced ATP hydrolysis, suggesting complete reversibility of the ATP synthase. Pi addition did not increase the amount of internal ATP hydrolysed (not shown).

![Fig. 1. Effect of CCCP on ATP synthesis and hydrolysis. Mitochondria (0.15 mg protein ml\(^{-1}\)) were incubated in 4 ml basal medium containing 0.6% ethanol and 0.2 mM (a) or 4 mM (b) Tris/phosphate. ATP synthesis was started by addition of ADP (400 nmol). As indicated, CCCP (5 \(\mu\)M) was added either before or 10 min after ADP addition. The Figure shows the amount of ATP in the absence (\(\star\)) or in the presence (\(\Diamond\)) of 5 \(\mu\)M-CCCP, the amount of ATP in the presence of 4 \(\mu\)g oligomycin (\(\triangledown\)) or 50 \(\mu\)M-mersalyl (\(\blacksquare\)) added 8 min after ADP addition and 2 min before CCCP, and the amount of ADP in the absence (\(\triangledown\)) or in the presence (\(\Delta\)) of 5 \(\mu\)M-CCCP.](image-url)
A TPase activity in yeast mitochondria

Fig. 2. ATP hydrolysis induced by valinomycin in a medium containing potassium phosphate. Mitochondria were incubated in basal medium containing 4 mM-potassium phosphate, 0.2 μg antimycin ml⁻¹ and 0.1 μg valinomycin ml⁻¹. Reaction was started by addition of 420 nmol ATP. ★, 50 μM-mersalyl added 1 min before ATP addition; ●, no mersalyl added.

Fig. 3. ATP hydrolysis induced by valinomycin in a medium containing potassium acetate or potassium chloride. Experimental conditions were identical to those described in the legend to Fig. 2 except that potassium phosphate was replaced by 4 mM-potassium acetate (●) or 4 mM-potassium chloride (★).

Table 1. Endogenous adenine nucleotide pool

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Total AdN</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>2.4 ± 0.6</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td>B</td>
<td>2.7 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>3.9 ± 0.7</td>
</tr>
<tr>
<td>C</td>
<td>0.6 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>2.0 ± 0.4</td>
<td>4.1 ± 0.9</td>
</tr>
</tbody>
</table>

Valinomycin-induced ATP hydrolysis. When added to medium containing K⁺, valinomycin collapsed Δψ, but not ΔpH. Valinomycin induced a rapid and nearly total hydrolysis of added ATP by mitochondria incubated in a medium containing 4 mM-potassium phosphate (Fig. 2). Mersalyl, added before ATP, completely inhibited ATP hydrolysis.

ATP hydrolysis in a medium containing potassium acetate instead of potassium phosphate was significant but lower than that observed in the presence of phosphate (Fig. 3). ATPase activity was, however, very low in a medium containing potassium chloride instead of potassium phosphate (Fig. 3).

Disruption of the permeability barrier by the use of detergent or by alkaline pH treatment. Kováč et al. (1968, 1972) reported that the oligomycin-sensitive ATPase activity in yeast mitochondria was maximal at alkaline pH and low at neutral pH, and that this activity was only slightly inhibited by atractyloside, an inhibitor of AdN translocation across the internal membrane, thus suggesting that the exposure of mitochondria to alkaline pH destroyed the permeability barrier. In our experiments, the ATPase activity was effectively higher at pH 8 than at pH 6-7 [0.8 and 0.1 μmol ATP hydrolysed min⁻¹ (mg protein)⁻¹, respectively]. Triton X-100 [0.75 mg (mg protein)⁻¹] increased the ATPase activity fourfold at pH 6-7. However, Triton inhibited the activity at pH 8. Addition of 10 mM-Tris/phosphate did not stimulate the ATPase activity either at alkaline pH or in the presence of Triton.

ATP⁻³²P exchange in state 4. The experiments described above were done in order to define the conditions which permit stimulation of the ATPase activity. To determine whether the ATP
Fig. 4. ATP–$^{32}$P exchange. Mitochondria were suspended in basal medium containing 0.6% ethanol and 4 mM-Tris/phosphate. ATP synthesis was started by adding 400 nmol ADP and was monitored by the bioluminescence method (●). In parallel experiments $^{32}$P was added after 10 min incubation such that the total external phosphate concentration was not significantly increased. ★, Incorporation of $^{32}$P into nucleotides measured as described in Methods; ▲, exchange reaction in the presence of 10 µg oligomycin ml$^{-1}$ added 2 min before $^{32}$P addition.

synthase worked in a reversible manner under conditions used for ATP synthesis, we compared the ATP synthesis and the ATP–$^{32}$P exchange (Fig. 4). This experiment clearly showed that all the ATP synthesized can be labelled by $^{32}$P, when the latter is added after the phosphorylation of ADP is achieved (state 4). It was verified that $^{32}$P was incorporated only in the γ-phosphate position of ATP by using the glucose phosphate/hexokinase test (not shown). The ATP–$^{32}$P exchange reaction was completely inhibited by oligomycin.

**DISCUSSION**

The ATP synthase in yeast mitochondria worked in a reversible manner under the experimental conditions that were used in the ATP synthesis experiments, since all the ATP pool was available for ATP–$^{32}$P exchange.

The lack of the CCCP-induced ATPase activity may be interpreted a priori in two ways: either as an inactivation of the ATPase complex under these conditions, or as an inaccessibility of external ATP to the hydrolysis site on the ATPase. The results of some experiments presented in this paper suggest that the ATPase is functional: (i) endogenous nucleotides were hydrolysed upon addition of CCCP; (ii) hydrolysis of exogenous ATP was greatly stimulated when the permeability barrier was disrupted either by alkaline pH or by detergent; (iii) ATPase activity was observed when the protonophore was replaced by potassium phosphate plus valinomycin.

Similar observations have been reported with plant mitochondria and with mammalian tumour mitochondria (see Introduction). Some studies have demonstrated that this anomalous behaviour was due, in part, to leakage of endogenous Mg$^{2+}$ (Jung & Hanson, 1975; Barbour & Chan, 1978; Hayashi et al., 1980b; Knowles & Kaplan, 1980). It has also been proposed that factors other than Mg$^{2+}$ leakage must be responsible for the lack of uncoupler-stimulated ATPase (Knowles, 1982). From the present work, it appears that such an effect cannot explain the low activity in yeast mitochondria since the endogenous ATP was hydrolysed and Mg$^{2+}$ addition did not stimulate the oligomycin-sensitive hydrolysis of exogenous ATP.

More probably, the low CCCP-induced ATPase activity reflects the presence of a permeability barrier to ATP. In energized mitochondria, for which Δψ is negative inside, influx of ADP$^{3−}$ and efflux of ATP$^{4−}$, catalysed by translocase, are favoured (Vignais, 1976; Klingenberg & Heldt, 1982). Thus, in hydrolysis (or ATP–$^{32}$P exchange) experiments, the ATP uptake can be limited either by Δψ or by the endogenous ADP pool size, or by both.

Table 1 shows that the internal ADN pool size was low when mitochondria were incubated in the absence of external ADP. Without external substrate or in the presence of uncoupler the percentage of the total ADN pool which was present as AMP, an unexchangeable nucleotide, was large. The low content of ADP may be responsible, in part, for a limitation of the ATPase
ATPase activity in yeast mitochondria

Fig. 5. (a) Electrogenic AdN exchange is compensated by a functional coupling between the CCCP-mediated electrogenic H⁺ influx and the Pₐ-carrier-mediated electroneutral H⁺ efflux. CCCP also equilibrates the ATPase mediated electrogenic H⁺ efflux. (b) Valinomycin-mediated K⁺ influx charge compensates both the AdN exchange and the ATPase mediated H⁺ efflux. The internal H⁺ concentration is maintained by Pₐ transport (or acetate diffusion). 1, Pₐ-carrier; 2, translocase; 3, H⁺-ATPase.

activity by ATP influx, as already demonstrated in new-born rabbit liver mitochondria (Rulfs & Aprille, 1982).

However, another important fact is the role of Pᵢ in stimulating the ATPase activity. There are several possible explanations for this effect.

(i) Internal Pᵢ stimulated the ATPase itself. It has been reported that some anions including phosphate can stimulate F₁ from mammalian mitochondria (Mitchell & Moyle, 1971; Ebel & Lardy, 1975). However, Fig. 1(b) shows that when Pᵢ was maintained inside mitochondria by addition of mersalyl, no stimulation of ATPase activity by uncoupler was observed. Moreover, 10 mM-Pₐ did not stimulate the activity of the isolated oligomycin-sensitive ATPase complex (not shown).

(ii) The phosphate flux may contribute to charge compensation during the ATP/ADP exchange. When the Pᵢ concentration was sufficiently high (Fig. 1 b) the CCCP-induced ATPase activity was high but it stopped 1 min after uncoupler addition. The effect of the addition of mersalyl before CCCP indicates the role of Pᵢ efflux in promoting hydrolytic activity; Pᵢ efflux may be necessary to balance charge due to ATP₄⁻/ADP₃⁻ exchange. This explanation does not implicate a mechanistic electrogenic Pᵢ efflux via a Pᵢ-carrier different from the H⁺/Pᵢ symporter but rather a charge equilibration between anions and H⁺ upon CCCP addition as shown in Fig. 5(a). This minimal interpretation is in agreement with the fact that the activation was time-dependent, since 1 min after uncoupler addition, mitochondria were Pᵢ-depleted (Rigoulet & Guérin, 1979).

Maximal ATP hydrolysis was obtained in the presence of valinomycin, K⁺ and an H⁺-donating anion (as Pᵢ or acetate). In this system, the total anion movement is charge-balanced by K⁺ influx as shown in Fig. 5(b). K⁺ influx in yeast mitochondria has been shown to occur under these conditions (Kovác et al., 1972). The requirement for a H⁺-donating anion is indicated by mersalyl inhibition and by the lack of ATPase activity in the presence of KCl, in contrast to the observation with tumour cell mitochondria (Knowles, 1982).

In conclusion, it appears that ATP synthase can work in a reversible manner in yeast mitochondria but that CCCP-induced ATPase activity is limited by ATP influx. One apparent reason for this limitation is the electrogenic nature of the ATP/ADP exchange. It is also likely that the low ADP pool size compared to that in mammalian mitochondria is a rate-limiting
The limitation of the ATP influx may prevent hydrolysis of the ATP synthesized in the cytosol.

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REFERENCES


