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# Enzyme turnover is essential for deactivation of $F_0F_1$-ATPase in plant mitochondria

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**Abstract**

In potato tuber mitochondria, ATPase deactivates immediately after treatment with an uncoupler or with polyoxyethylene 9-lauryl ether (Lubrol), a non-ionic detergent. Deactivation was completely prevented by another non-ionic detergent, lauryldimethylamine oxide (LDAO). LDAO also induced slow reactivation of inactive ATPase formed in deenergized mitochondria. Freezing of the active state by LDAO was used to study the process of ATPase deactivation following deenergization in intact mitochondria. Deactivation was slowed down by carboxyatractyloside (CATR), which prevents ATP import into the matrix, and by ATPase inhibitors. ATP hydrolysis was also triggered by Lubrol with CATR-treated mitochondria. The initial rate was close to the capacity for ATP synthesis but rapidly decayed. The rate of decay increased with the concentration of MgATP and no decay was observed in the presence of EDTA. The following conclusions were drawn. (1) Deenergization in itself is not sufficient for ATPase deactivation in plant mitochondria: enzyme turnover is also required. The probability of one enzyme to be deactivated at each turnover is much higher in potato tuber than in pea leaf organelles. (2) Enzyme turnover probably shifts the IF1-$F_1$ complex from an active to an inactive form; the rate of deactivation indeed does not seem to be controlled by the binding of the inhibitory peptide. (3) The short-term effect (protection) and the long-term effect (reactivation) of LDAO on $MF_0MF_1$ may tentatively be used to titrate the activated versus total amounts of these enzymes in cells.

*Keywords:* ATPase, $F_0F_1$-; Enzyme activation; Plant mitochondrion; Detergent; ATP hydrolysis; Proton gradient

## 1. Introduction

In chloroplasts, mitochondria and some bacteria, the electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$ has an activatory effect on the $F_0F_1$ $H^+$-ATPase in addition to its protonmotive role (reviews [1–5]). Deactivation of ATPase following collapse of $\Delta\tilde{\mu}_{H^+}$, which prevents wasteful ATP hydrolysis, is considered as an adaptive response to normal or pathological physiological situations: darkness in the case of chloroplasts, anaerobiosis or uncoupling for animal mitochondria (reviews for mitochondria: [4,5]). In intact plant mitochondria from different species and tissues, ATP hydrolysis under uncoupled conditions was generally found considerably lower than the capacity for ATP synthesis [6–12]. In potato tuber mitochondria, ATPase was virtually inactive as soon as the inner membrane was discharged with an uncoupler [13]. By contrast, pea leaf preenergized mitochondria hydrolysed ATP at high rates upon addition of an uncoupler, but this activity decayed in tens of seconds [13]. Since both types of organelle catalyze ATP synthesis at comparable rates, we previously proposed that the ATPase, activated by $\Delta\tilde{\mu}_{H^+}$, decays much more rapidly in mitochondria isolated from potato tubers than from pea

Abbreviations: CATR, carboxyatractyloside; $\Delta\tilde{\mu}_{H^+}$, transmembrane difference in proton electrochemical potential (electrochemical proton gradient); $\Delta\psi$, transmembrane electrical potential difference (membrane potential); EDTA, ethylenediaminetetraacetic acid; $F_0$, membranous sector of the $H^+$-ATPase; $F_1$, extrinsic, catalytic sector of the $H^+$-ATPase; IF1, inhibitory peptide of the mitochondrial $H^+$-ATPase; LDAO, lauryldimethylamine oxide; Lubrol, polyoxyethylene 9-lauryl ether (polidocanol); PEP, phospho*enol*pyruvate; $TPP^+$, tetraphenylphosphonium cation; Tricine, *N*-(2-hydroxy-1,1-bis (hydroxymethyl)ethyl)glycine.

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leaves [13]. This was confirmed by experiments in which the activated state was frozen by LDAO addition to energized mitochondria. This detergent triggered a very high rate of ATP hydrolysis, but only when ATPase had been previously activated by $\Delta\tilde{\mu}_{H^+}$ [14]. A negligible activity was triggered by LDAO in deenergized potato tuber mitochondria. Another non-ionic detergent, Lubrol, failed to reveal any ATP hydrolysis in potato tuber mitochondria [14].

However, $\Delta\tilde{\mu}_{H^+}$ is not the only effector of ATPase regulation. Tight binding of ADP inhibits the enzyme, isolated or bound to deenergized membranes, at least in the case of thylakoids [15,16] and animal mitochondria [17,18]. In mitochondria from animal tissue [19], plant tissue [20] and yeast [21], a key role is played by an endogenous inhibitory peptide called IF1. Binding of one IF1 per ATPase blocks ATP hydrolysis [22]. In animal mitochondria or submitochondrial particles, $\Delta\tilde{\mu}_{H^+}$ activates the ATPase by promoting IF1 dissociation [23–30] or by inducing a transition of inactive $F_1$-IF1 complex to an active state [31]. The presence of MgATP, or more likely the existence of a specific intermediate state of the catalytic cycle, seems to be required for IF1 rebinding and/or inactivation of the $F_1$-IF1 complex [23,32–35] in the absence of $\Delta\tilde{\mu}_{H^+}$. The role of MgATP could not be investigated until now in isolated plant mitochondria, because the presence of nucleotides is required in these organelles in order to obtain complete energization [7,36–38].

In this report, we have investigated the role of MgATP and the catalytic turnover in the deactivation of ATPase following deenergization in plant mitochondria. The effect of different inhibitors on the rate of ATPase deactivation was also studied, using the property of LDAO to reverse their effect on the catalytic cycle [14]. All the data showed that in plant mitochondria ATPase deactivation following deenergization is strictly dependent on the enzyme turnover. The study was focused on potato tuber mitochondria, but some data obtained with pea leaf mitochondria suggest that only quantitative aspects differentiate the properties of the two types of ATPase.

## 2. Materials and methods

### 2.1. Mitochondria preparation and storage

Extraction and purification of mitochondria from potato (*Solanum tuberosum* L.) tubers or from pea (*Pisum sativum* L.) leaves were carried out at 4° C as previously described [39,13]. Purified mitochondria were stored before use on ice in a medium containing 0.4 M mannitol, 5 mM $MgCl_2$, 30 mM KCl, 0.1% (w/v) bovine serum albumin, 2 mM potassium phosphate and 2 mM Tricine (pH 7.5), at a protein concentration of 10–20 mg $ml^{-1}$, determined using the Bradford method [40]. The activity remained fully stable for at least 2 days.

### 2.2. 'Conditioning' of mitochondria

Mitochondria were suspended, at a concentration of 0.1–0.2 mg protein per ml, in a medium containing, unless otherwise indicated, 0.4 M mannitol, 1 mM $MgCl_2$, 30 mM KCl, 0.1% (w/v) bovine serum albumin, 2 mM potassium phosphate, and Tricine buffer (2 mM for pH-metric measurements, 20 mM for the experiments with the ATP-regenerating system), pH 8.0. All experiments were run at 25° C. Sodium succinate (10 mM) was added, then two successive ADP injections (50 $\mu$M each) were made, so that mitochondria experienced two State 3–State 4 [41] transitions. The duration of the conditioning stage was 5 min [13].

### 2.3. Measurement of ATP hydrolysis with LDAO and ATP-regenerating system

10 to 20 $\mu$l of conditioned mitochondria were taken up and put into a spectrophotometric cuvette containing 1 ml of the conditioning medium supplemented with 2 mM $MgCl_2$, 2 mM ATP, 1 mM PEP, 0.3 mM NADH, 15 units/ml pyruvate kinase, 15 units/ml lactate dehydrogenase, and 0.3% LDAO (pH 8.0, 25° C). LDAO instantaneously disrupted the membranes [14], and ATP hydrolysis was monitored by NADH oxidation at 340 nm. Solubilized mitochondria did not oxidize NADH in the absence of ATP. Experiments with deenergized mitochondria were carried out by adding valinomycin and nigericin, at the indicated concentrations, at the end of the conditioning stage. No ATP was hydrolyzed unless LDAO was added. When indicated, inhibitors at saturating concentrations were added in the incubation medium 5 min before the uncoupler. In some cases, mitochondria were incubated with LDAO (0.3%) for different times before being injected into the assay medium.

### 2.4. Triggering and measurement of ATP hydrolysis with Lubrol and the pH-metric technique

The 3 ml sample was conditioned and assayed (pH 8.0, 25° C) into the closed, stirred and thermostatted chamber of the apparatus previously described [14]. ATP synthesis and hydrolysis were deduced from the consumption or production of scalar $H^+$ ions [42]. pH variations were detected with a fast and sensitive glass electrode, and the suspension was titrated by HCl after each run. The activity of the respiratory chain and the membrane potential were simultaneously controlled, respectively by $O_2$ consumption (Clark-type electrode) and $TPP^+$ uptake [43] (laboratory-made specific electrode). After mitochondrial conditioning, ATP hydrolysis was triggered in three different ways: (1) by addition of Lubrol (0.02% w/v) to mitochondria in the presence of MgATP, at different times after addition of an uncoupler; (2) by giving ATP at different times after Lubrol addition; (3) by adding $MgCl_2$ and ATP at differ-

ent times after Lubrol addition, magnesium present in the assay being previously removed by EDTA. Protocols are detailed in the text and in the legends of the figures. The different additions used to trigger ATP hydrolysis resulted in small transient pH shifts, positive or negative. This led to us discard the first seconds of the kinetics for analysis of ATP hydrolysis. The duration of the eliminated section was determined from experiments with oligomycin 5 $\mu$g (mg protein)$^{-1}$ (see Fig. 5).

### 2.5. Rates of ATP hydrolysis

For both types of measurement (spectrophotometric or pH-metric), initial rates were estimated on the direct analog trace, and instantaneous rates were calculated by taking the first derivative of the digitalized trace.

### 2.6. Reagents

All reagents were of analytical grade. Valinomycin, nigericin, polyoxyethylene 9-lauryl ether (Lubrol), oligomycin, carboxyatractyloside, ADP (ref. A-5394) and ATP (ref. A-2754) were purchased from Sigma. Lauryldimethylamine oxide (LDAO) from Fluka (30% in water) was treated by small amounts of catalase to eliminate peroxides.

## 3. Results

### 3.1. Time-dependence of ATP hydrolysis in the presence of detergent lauryldimethylamine oxide (LDAO)

Fig. 1a, shows the time-course of ATP hydrolysis by mitochondria disrupted by LDAO in the presence of the ATP-regenerating system. With pre-energized mitochondria (top trace), the activity was initially high and decreased with time. In mitochondria preincubated with an uncoupler (bottom trace), the initial activity was very low, as previously reported [14], but increased with time. In the presence of LDAO, the two activities finally reached close values (compare the slopes at the end of the kinetics). In deenergized mitochondria, time-dependent ATPase deactivation was probably superimposed to the stimulatory effect of LDAO. Since this deactivation only occurs in the presence of a high ATP concentration (mM range) [14], the long-term effect of LDAO on the ATPase of deenergized mitochondria was investigated with an ATP concentration as low as possible (100 $\mu$M, synthesized during the conditioning process). For this purpose, mitochondria were conditioned as described under Materials and methods, deenergized with nigericin + valinomycin for 10 min, then incubated with LDAO for different times and finally assayed for ATP hydrolysis. Fig. 1b shows how the initial rate of ATP hydrolysis increased with the time of incubation with LDAO. After about 40 min, the activity was the same as that triggered on preenergized mitochondria. Moreover, after correction for ATP-dependent deactivation (explained in legend of Fig. 1), the induction of ATPase activity by LDAO in the presence of high ATP concentration had the same time-dependency (Fig. 1b, dotted trace, data from Fig. 1a).

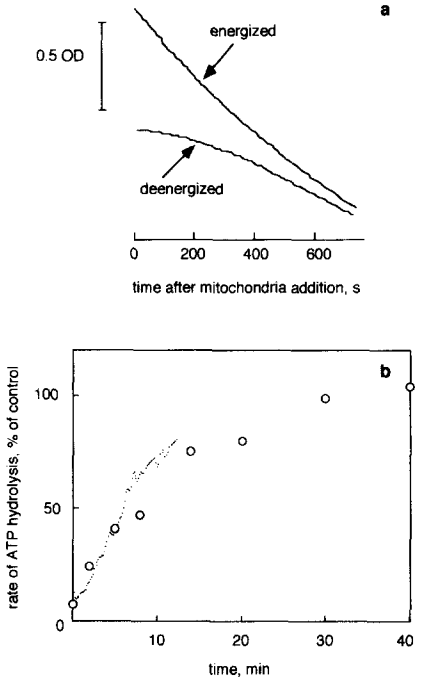


Fig. 1. Time-course of LDAO-triggered ATP hydrolysis with potato tuber mitochondria. Effect of incubation with LDAO. Conditions as described under Materials and methods, spectrophotometric measurement. (a) Absorbance at 340 nm as a function of the time. Top trace, energized mitochondria; bottom trace, deenergized mitochondria. (b) Initial rate of ATP hydrolysis (○) as a function of the time of preincubation of deenergized potato tuber mitochondria with LDAO. The instantaneous normalized rates (dotted trace) plotted on the graph b were obtained as follows: (1) taking the first derivatives of the two curves shown in a; (2) dividing the rates so obtained from the bottom trace (deenergized) by those obtained from the top trace (energized). Protein concentrations: conditioning, 0.22 mg ml$^{-1}$; assay, 2.2 $\mu$g ml$^{-1}$ (a) or 4.4 $\mu$g ml$^{-1}$ (b). Uncoupler concentrations: valinomycin, 0.15 $\mu$g (mg protein)$^{-1}$; nigericin, 0.9 $\mu$g (mg protein)$^{-1}$. Activity of the control (energized mitochondria): 8.3 $\mu$mol ATP/mg protein per min.

To summarize, LDAO prevents rapid inactivation of ATPase induced by deenergization [14] and slowly reactivates the enzyme which was previously deactivated. High MgATP concentration induces a slow deactivation in the presence of LDAO.

### 3.2. Effect of inhibitors on the uncoupler-induced ATPase deactivation

The question which arose was whether ATP affects the deactivation of ATPase by binding to a regulatory site, or if its effect is related to the enzyme turnover. Therefore, we have studied the effect of some inhibitors on the decay of the activity in uncoupled mitochondria, as revealed by

LDAO. This was made possible by the property of LDAO to reverse the inhibitory effect of $F_0$-blockers on ATPase activity, as previously reported [14]. Conditioned mitochondria (containing 100 μM ATP) were incubated with valinomycin + nigericin for various times, then aliquots were put in the assay medium containing LDAO. Fig. 2 shows that deactivation was almost complete in a few seconds, in accordance with our previous observations [14]. Oligomycin considerably slowed down the decay: 20 min, instead of a few seconds, were necessary to lose 50% of the activity. Venturicidin also significantly slowed down the deactivation: the half-decay was reached in about 1 min. Both inhibitors were used at concentrations largely sufficient to decrease ATP synthesis below detection level (more than 98% inhibition).

ATPase turnover may also be prevented by carboxyatractyloside (CATR), which inhibits the ATP/ADP antiporter. Fig. 2 shows that, like ATPase inhibitors, CATR at saturating concentration slowed down ATPase deactivation in uncoupled mitochondria. These data indicate that ATP hydrolysis plays an important role in the mechanism of uncoupler-induced ATPase deactivation.

### 3.3. ATP hydrolysis in the absence of LDAO

Until now, LDAO addition was the only way to trigger significant ATP hydrolysis in potato tuber mitochondria, but this detergent puts the enzyme into a state which is probably far from the physiological conformation [13,14].

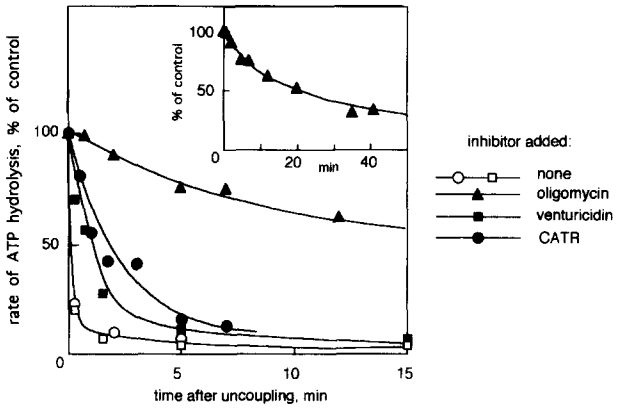


Fig. 2. Initial rate of LDAO-triggered ATP hydrolysis as a function of the time of deenergization of potato tuber mitochondria. Effect of inhibitors. Conditions as described under Materials and methods, spectrophotometric measurement, two different mitochondrial preparations. Inhibitor was added 5 min before uncoupler. Protein concentrations: conditioning, 0.22 mg $ml^{-1}$ (□, ■, ▲) or 0.37 mg $ml^{-1}$ (○, ●); assay, 2.2 μg $ml^{-1}$ (□, ■, ▲) or 7.4 μg $ml^{-1}$ (○, ●). Uncoupling mixture concentrations: valinomycin, 0.15 μg (mg protein)$^{-1}$ plus nigericin, 0.9 μg (mg protein)$^{-1}$ (□, ■, ▲) or valinomycin 0.1 μg (mg protein)$^{-1}$ plus nigericin 0.5 μg (mg protein)$^{-1}$ (○, ●). Activity of the control: 8.3 μmol ATP/mg protein per min (□, ■, ▲) or 11 μmol ATP/mg protein per min (○, ●). Additions: □, ○, without inhibitor; ■, venturicidin 18 μg (mg protein)$^{-1}$; ▲, oligomycin 4.6 μg (mg protein)$^{-1}$; ■, carboxyatractyloside 2.3 μg (mg protein)$^{-1}$. (Inset) ▲, data with oligomycin obtained on a longer time range.

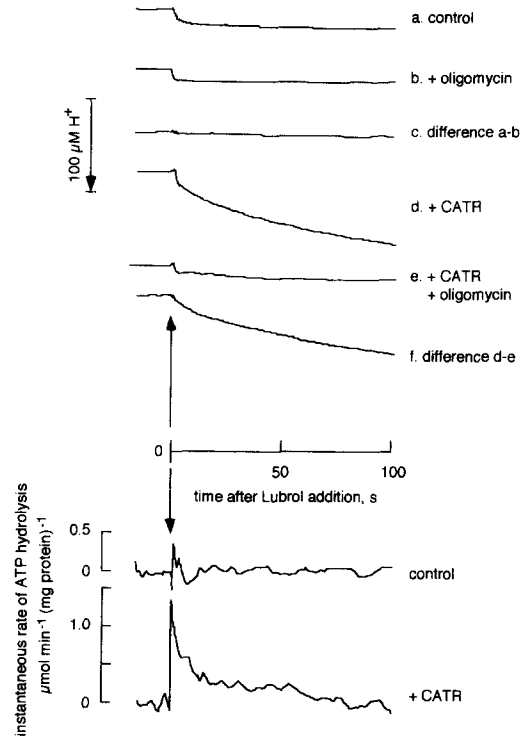


Fig. 3. Time-course of ATP hydrolysis triggered by Lubrol addition to energized potato tuber mitochondria. Effect of carboxyatractyloside. Conditions as described under Materials and methods, pH-metric measurement. Lubrol, 0.02% (w/v). Upwards, ATP synthesis; downwards, ATP hydrolysis. Protein concentration, 0.14 mg $ml^{-1}$. Traces a–b and d–e, pH recordings converted into equivalent-$H^+$ by HCl titration. Traces c and f were obtained by subtracting b to a and e to d, respectively. Bottom traces, instantaneous rates of ATP hydrolysis vs. time, obtained by the first derivative of trace c (without CATR) and f (with CATR). CATR (4 μg (mg protein)$^{-1}$) was added 10 s before ATP (traces d–e). Traces b,e: oligomycin (5 μg (mg protein)$^{-1}$) was added 5 s before the second ADP injection, which fully suppressed the second State 3–State transition.

At variance with LDAO, Lubrol, which also disrupts mitochondria, does not modify the ATPase [44,14]. In pea leaf mitochondria, Lubrol does not reverse the effect of ATPase inhibitors [14], but neutralizes the effect of CATR on the ATP/ADP antiporter by disrupting the membrane. Since CATR slowed down the deenergization-induced decay of the LDAO-revealed ATPase activity, we have tried to trigger ATPase activity in potato tuber mitochondria treated by CATR and then disrupted by Lubrol. After conditioning of mitochondria, ATP (1 mM) and Lubrol were added. Fig. 3 shows that no significant ATP hydrolysis was induced by Lubrol without CATR, as previously reported [14] (compare trace a, control, with trace b obtained after preincubation with oligomycin) [1]. When CATR was added before ATP, a significant oligomycin-sensitive

[1] Depending on mitochondrial preparation, the initial rate actually varied from almost zero to 30% of the rate of ATP synthesis in State 3 (not shown), but it decayed in a very short time, preventing any accurate estimation (see Fig. 4).

ATP hydrolysis was triggered by Lubrol (trace d and f). This activity was higher than the rate of ATP synthesis in State 3, and decayed in tens of seconds after Lubrol addition. Instantaneous rates of ATP hydrolysis vs. time were plotted in the bottom of Fig. 3.

We have studied the time-dependence of the deactivation of Lubrol-revealed ATP hydrolysis in potato tuber mitochondria deenergized by valinomycin + nigericin. Fig. 4 shows the initial rate of ATP hydrolysis as a function of the time separating uncoupling and Lubrol addition. Without CATR, the rate, already low, became completely insignificant a few seconds after uncoupling. In the presence of CATR added together with the uncoupler, the activity further revealed by Lubrol was high and much more resistant to deenergization, the half-decay requiring almost 2 min.

### 3.4. Effect of MgATP on ATPase deactivation in disrupted mitochondria

It is not possible to energize potato tuber mitochondria to the maximal extent without added nucleotides. Therefore, in order to study the ATPase deactivation in the absence of substrate (i.e., MgATP but not free ATP) we removed $Mg^{2+}$ by adding EDTA in excess at the end of the conditioning process (after CATR addition). Then, membranes were disrupted by Lubrol, and MgATP (with magnesium in excess) was added at different times to trigger ATP hydrolysis. Fig. 5 shows that the ATPase activity measured (squares, curve 1) was fully stable for at least 10 min. This demonstrates that removal of magnesium, which prevents ATP hydrolysis, also completely prevented the deactivation of ATPase in disrupted mitochondria.

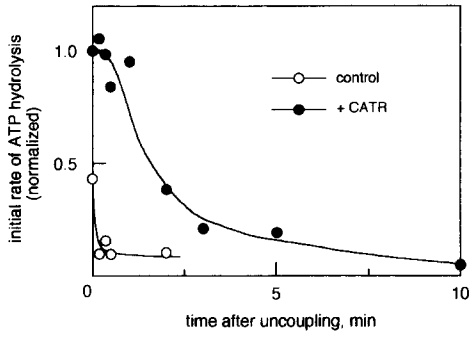


Fig. 4. Initial rate of ATP hydrolysis as a function of the time between uncoupler and Lubrol additions to potato tuber mitochondria. Effect of carboxyatractyloside. Conditions as described under Materials and methods, pH-metric measurement. Protein concentration, 0.15 mg $ml^{-1}$. ATP hydrolysis was triggered by adding Lubrol (0.02% w/v) to mitochondria which were previously conditioned, put in presence of 1 mM ATP, and deenergized for the indicated time with valinomycin (0.22 $\mu$g (mg protein)$^{-1}$) and nigericin (1.1 $\mu$g (mg protein)$^{-1}$). ○, no CATR; ●, CATR 5.5 $\mu$g (mg protein)$^{-1}$). For initial rates measurements, the first 3 s of the kinetics were discarded in order to eliminate the artifact due to Lubrol addition. 100% activity (zero time, CATR): 1.7 $\mu$mol ATP/mg protein per min.

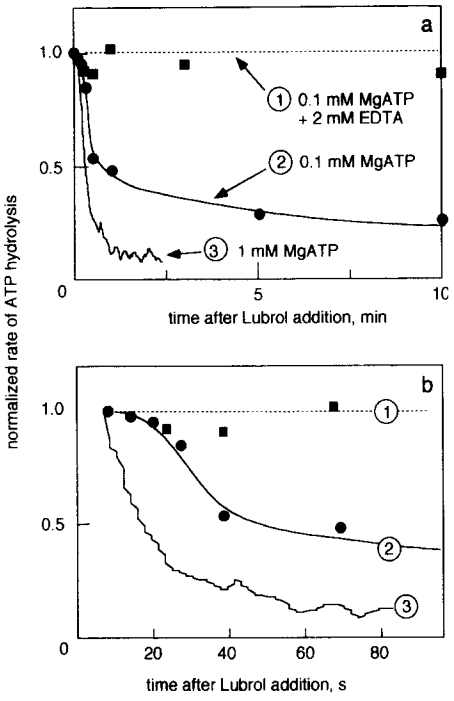


Fig. 5. Rates of ATP hydrolysis as a function of the time between Lubrol and ATP additions to potato tuber mitochondria. Conditions as described under Materials and methods, pH-metric measurement. Conditions indicated in (a) under labels 1–2 refer to those encountered before triggering ATP hydrolysis. Discrete values (curves 1,2), initial rates; continuous trace (curve 3), instantaneous rate, computed from the first derivative of the pH-metric recording. Curve 1 (■): ATP hydrolysis triggered by addition of 3 mM $MgCl_2$ + 1 mM ATP to mitochondria which were previously conditioned, treated with CATR (6.9 $\mu$g (mg protein)$^{-1}$), magnesium-depleted by the addition of EDTA (2 mM), and then treated with Lubrol (0.02% w/v) for the indicated times. Curve 2 (●): ATP hydrolysis triggered by addition of 1 mM ATP to mitochondria previously conditioned, treated with CATR (5.2 $\mu$g (mg protein)$^{-1}$), then treated with Lubrol for the indicated times. Curve 3 (continuous trace): monitoring of the instantaneous rate of ATP hydrolysis, triggered by Lubrol addition to mitochondria previously conditioned, treated with CATR (5.2 $\mu$g (mg protein)$^{-1}$), and after addition of 1 mM ATP. The first 6 s of each kinetics were discarded, to eliminate the artifacts due to the injections of $MgCl_2$ + ATP, ATP or Lubrol. a, time range from 0 to 10 min. b, same data with an expanded time scale. Protein concentrations, 0.12 mg protein $ml^{-1}$ (1) and 0.16 mg $ml^{-1}$ (2–3). 100% activities (6 s after Lubrol): 0.9 $\mu$mol ATP/mg protein per min (1), 0.8 $\mu$mol ATP/mg protein per min (2–3).

In the absence of EDTA, after conditioning of mitochondria, the concentration of MgATP was 100 $\mu$M. Under these conditions, after treatment by CATR, the activity decayed with the time separating Lubrol and ATP (1 mM) additions (Fig. 5, circles, curve 2). The deactivation seemed somewhat faster than that observed in uncoupled mitochondria (compare with Fig. 4), but a significant residual activity remained a long time after Lubrol addition. This biphasicity is probably due to the presence of MgATP in the seconds following membrane disruption

(leading to ATPase turnover and deactivation), but not a few minutes later.

Lastly, a trace of the instantaneous rate of ATP hydrolysis, similar to that of Fig. 3, bottom, was also plotted to show the deactivation during ATP hydrolysis, with 1 mM MgATP (Fig. 5, continuous curve, labeled 3). The decay was clearly faster than with 100 $\mu$M MgATP (Fig. 5b, expanded time scale). The time-course of ATP hydrolysis did not change anymore with MgATP concentrations ranging from 1 to 7 mM (data not shown), so 1 mM MgATP was saturating both for the enzyme turnover and deactivation. In addition, free magnesium (0 to 5 mM) and free ATP (0 to 5 mM) did not alter the kinetics (not shown).

### *3.5. Deactivation of ATPase in deenergized pea leaf mitochondria*

Some experiments (results not shown) were also done with pea leaf mitochondria, in which the ATPase deactivates more slowly and less completely than in potato tuber mitochondria [13,14,45]. Deenergization of pea mitochondria for 15 min with an uncoupler resulted in a loss of 45% of the LDAO-revealed activity, in accordance with previous results [14]. Oligomycin (8 $\mu$g (mg protein)$^{-1}$) or sodium azide (5 mM), added before uncoupling, fully prevented the deactivation. With venturicidin (30 $\mu$g (mg protein)$^{-1}$) the loss of activity in 15 mn was reduced from 45% to 20%. CATR (6 $\mu$g (mg protein)$^{-1}$) almost completely prevented the loss of LDAO-revealed as well as Lubrol-revealed ATPase activities.

## 4. Discussion

### *4.1. Enzyme turnover and ATPase deactivation in deenergized plant mitochondria*

In previous studies [14,45], we have shown that the low rate of ATP hydrolysis in intact potato tuber mitochondria could not be attributed to a slow transport of substrates or a low turnover rate of ATPase, but only to its deactivation after deenergization. In this work, ATPase regulation was studied in intact organelles, using detergents to reveal active/inactive state transitions of the enzyme. Removal of substrate (MgATP), inhibition of ATP hydrolysis and blocking of nucleotide transport suppressed or slowed down the deactivation. This indicated that catalytic turnover of ATPase is necessary for its deactivation in deenergized plant mitochondria. Although their effect on ATP synthesis is the same, venturicidin and oligomycin (both inhibitors of the proton flow through $F_0$) did not protect the ATPase from deactivation with the same efficiency (Fig. 2). Oligomycin may block the channel more completely than venturicidin [46]. Alternatively, since venturicidin is not an irreversible inhibitor [47], the statistical period when the enzyme is inhibited could be long enough to fully prevent ATP synthesis, but not enough to avoid the formation of a fastly-deactivating intermediate. Lastly, the possibility cannot be excluded that the mechanism by which oligomycin inhibits the ATPase does not simply consist of interrupting the proton flow [48]. Anyway, these data indicate that even very slow ATPase turnover is sufficient for operation of the mechanism of deactivation.

The essential role of enzyme turnover in deactivation was recognized early on for $F_1$ isolated from animal mitochondria (but not membrane-bound), where the catalytic turnover and the rate of deactivation had grossly the same ATP concentration dependency [49]. Some microscopic state formed during ATP hydrolysis probably quickly deactivates in the absence of $\Delta\tilde{\mu}_{H^+}$ [26,49,22]. In deenergized thylakoids, phloridzin and venturicidin, used at non-saturating concentrations, slowed down the deactivation of the thiol-reduced ATPase [50]. In animal submitochondrial particles, oligomycin and efrapeptin blocked the energy-dependent release of IF1, but aurovertin stimulated it [26]. These opposite effects could be due to the blocking of different steps of the catalytic cycle, leading to different distributions of microscopic states.

It was extensively reported [15–17] that MgADP forms a tight binding inactive complex with $F_1$. In our experiments this mechanism probably did not play an important role in ATPase deactivation. Indeed, CATR protected the enzyme from deactivation, although its effect normally leads to ADP accumulation (at the expense of ATP) within the matrix.

### *4.2. Effect of detergents*

Two different detergents were used in this work to instantaneously disrupt mitochondria: LDAO and Lubrol. LDAO was known to stimulate the activity of $F_1$ ATPase isolated from animal [51] or plant [52,53] mitochondria, from *Escherichia coli* [54] and from the thermophilic bacterium *Bacillus* PS3 [55]. In animal mitochondria, one of the effects of LDAO could be to reverse the inhibitory effect of IF1 [51]. In plant mitochondria, LDAO had different effects: it increased the turnover rate of active ATPase [14], prevented the deactivation of ATPase [14] and induced reactivation of the inactive enzyme formed in intact mitochondria after deenergization (Fig. 2). LDAO then appears to be a potentially interesting tool to study the mitochondrial ATPase in cells: short incubation would reveal the $\Delta\tilde{\mu}_{H^+}$-activated ATPases, and long incubation the total amount of enzymes.

The second detergent used in this work was Lubrol, more precisely polyoxyethylene 9-lauryl ether, which has properties similar to Lubrol-WX, used in previous studies [34,44]. Contrary to LDAO, this detergent has no significant effect on the ATPase itself. In the present report, Lubrol allowed detection of high rates of ATP hydrolysis, but only when used in association with some pretreatments: EDTA or CATR, to transiently deplete the ATPase

from MgATP. The association of CATR (to inhibit nucleotide transport) and Lubrol (to break membranes) may seem paradoxical and requires some comments. To simply explain the protective effect of CATR, one actually should assume that the sequence of events upon Lubrol addition (which cannot be time-resolved in our experiments) is the following: (1) $\Delta\tilde{\mu}_{H^+}$ collapse due to leaks for protons and small ions; (2) hydrolysis of ATP of the matrix (high concentration but low amount); (3) stopping or slowing down of ATP hydrolysis due to ATP exhaustion and ADP accumulation (CATR present); (4) more complete membrane disruption and hydrolysis of medium ATP; (5) deactivation (lifetime ≈ 10 s with saturating MgATP, i.e., in mM range).

### 4.3. ATPase deactivation and IF1

In solubilized or membrane-bound animal MF1, the inactivating effect of MgATP was often discussed in relation with the inhibitory peptide IF1: in some reports, MgATP prevented the energy-induced IF1 release in beef heart submitochondrial particles [23], or accelerated its binding or its inhibitory effect in deenergized particles [32,33]. In other reports, MgATP actually shifted the $F_1$-IF1 complex, membrane-bound [34] or isolated [35], from an active to an inactive state. In all cases, MgATP or enzyme turnover was necessary to induce inactivation by IF1. Other nucleotide triphosphates were able to promote the inhibition by IF1 of the isolated [35] or membrane-bound [33] ATPase, although ATP was the most effective. In some special conditions (preincubation with ADP or free $Mg^{2+}$), a progressive inhibition of isolated $MF_1$ occurred during the catalytic turnover, independently of the presence of IF1 [22,56–58]. Whatever it may be, since an inhibitory peptide of the plant mitochondria ATPase has been isolated [20], its role in the deactivation of the enzyme is probable here.

We have no direct indication on the role of IF1, but it seems unlikely that binding of a soluble matricial peptide controlled the rates of deactivation in our experiments. The decay was indeed faster in disrupted membrane (Fig. 5, curve 2) than in uncoupled mitochondria (Fig. 4, black circles), although membrane solubilization is expected to prevent IF1 rebinding by diluting it at least 1000-fold. It is more probable that IF1 dissociated by membrane energization quickly rebound upon Lubrol addition, before the membrane became leaky to nucleotides (and later, to IF1 itself). The subsequent decay of activity would be due to the turnover-related transition of the $F_1$-IF1 complex from an active to an inactive form [34,49].

### 4.4. Conclusion

The inactivation of the ATPase is more difficult to study in plant mitochondria than in animal mitochondria, due to the necessary conditioning of organelles in the presence of nucleotides. It seems, however, that this process obeys the same general laws in plant and animal mitochondria, even though some quantitative differences (IF1 content or affinity, rate constants) may exist in various tissues. A good example is given by the two kinds of plant mitochondrion that we have studied. In potato tubers and pea leaves, the deactivation pattern of the ATPase is similar in the sense that it requires enzyme turnover in both cases. However, the probability of being deactivated during one turnover is much higher in the ATPase of potato tubers than in that of pea leaves. The reasons for such a difference remain to be elucidated and will be the subject of future investigations. Finally, detergents used in this work allow us to discriminate $\Delta\tilde{\mu}_{H^+}$-activated and inactive forms of mitochondrial ATPase in a set of different conditions. For this reason, we suggest that they can be used as tools to study the status of this enzyme in living cells, in relation to physiological or pathological situations.

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## References

[1] Schlodder, E., Gräber, P. and Witt, H.T. (1982) in Topics in Photosynthesis (Barber, J., ed.), Vol. 4, pp. 105–175, Elsevier, Amsterdam.
[2] Schwerzmann, K. and Pedersen, P.L. (1986) Arch. Biochem. Biophys. 250, 1–18.
[3] Chernyak, B.V. and Kozlov, I.A. (1986) Trends Biochem. Sci. 11, 32–35.
[4] Harris, D.A., and Das, A.M. (1991) Biochem. J. 280, 561–573.
[5] Rouslin, W. (1991) J. Bioenerg. Biomembr. 23, 873–888.
[6] Blackmon, W.J. and Moreland, D.E. (1971) Plant Physiol. 47, 532–536.
[7] Jung, D.W. and Hanson, J.B. (1973) Arch. Biochem. Biophys. 158, 139–148.
[8] Jung, D.W. and Hanson, J.B. (1973) Biochim. Biophys. Acta 325, 189–192.
[9] Passam, H.C. and Palmer, J.M. (1973) Biochim. Biophys. Acta 305, 80–87.
[10] Takeuchi, Y. (1975) Biochim. Biophys. Acta 376, 505–518.
[11] Jung, D.W. and Laties, G.G. (1976) Plant Physiol. 57, 583–588.
[12] O'Rourke, J.F. and Wilson, S.B. (1992) J. Exp. Bot. 43, 1507–1514.
[13] Valerio, M., Haraux, F., Gardeström, P. and Diolez, P. (1993) FEBS Lett. 318, 113–117.
[14] Valerio, M., Diolez, P. and Haraux, F. (1993) Eur. J. Biochem. 216, 565–571.
[15] Strotmann, H. and Bickel-Sandkötter, S. (1977) Biochim. Biophys. Acta 460, 126–135.

[16] Bar-Zvi, D. and Shavit, N. (1982) Biochim. Biophys. Acta 681, 451–458.
[17] Fitin, A.F., Vasilyeva, E.A. and Vinogradov, A.D. (1979) Biochem. Biophys. Res. Commun. 86, 434–439.
[18] Chernyak, B.V. and Khodjaev, E.Yu. (1989) FEBS Lett. 254, 79–82.
[19] Pullman, M.E. and Monroy, G.C. (1963) J. Biol. Chem. 238, 3762–3769.
[20] Norling, B., Tourikas, C., Hamasur, B. and Glaser, E. (1990) Eur. J. Biochem. 188, 247–252.
[21] Satre, M., De Jerphanion, M.-B., Huet, J. and Vignais, P.V. (1975) Biochim. Biophys. Acta 387, 241–255.
[22] Gomez-Fernandez, J.C. and Harris, D.A. (1978) Biochem. J. 176, 967–975.
[23] Van de Stadt, R.J., De Boer, B.L. and Van Dam, K. (1973) Biochim. Biophys. Acta 292, 338–349.
[24] Harris, D.A., Von Tscharner, V. and Radda, G.K. (1979) Biochim. Biochim. Acta 548, 72–84.
[25] Schwerzmann, K. and Pedersen, P.L. (1981) Biochemistry 20, 6305–6311.
[26] Power, J., Cross, R.L. and Harris, D.A. (1983) Biochim. Biophys. Acta 724, 128–141.
[27] Husain, I. and Harris, D.A. (1983) FEBS Lett. 160, 110–114.
[28] Klein, G. and Vignais, P.V. (1983) J. Bioenerg. Biomembr. 15, 347–362.
[29] Husain, I., Jackson, P.J. and Harris, D.A. (1985) Biochim. Biophys. Acta 767, 64–74.
[30] Lippe, G., Sorgato, M.C. and Harris, D.A. (1988) Biochim. Biophys. Acta 933, 1–11.
[31] Dreyfus, G., Gòmez-Puyou, A. and Tuena de Gòmez-Puyou, M. (1981) Biochem. Biophys. Res. Commun. 100, 400–406.
[32] Lippe, G., Sorgato, M.C. and Harris, D.A. (1988) Biochim. Biophys. Acta 933, 12–21.
[33] Cintròn, N.M., Hullihen, J., Schwerzmann, K. and Pedersen, P.L. (1982) Biochemistry 21, 1878–1885.
[34] Chernyak, B.V., Dukhovich, V.F. and Khodjaev, E.Yu. (1991) Arch. Biochem. Biophys. 286, 604–609.
[35] Galante, Y.M., Wong, S.-Y. and Hatefi, Y. (1981) Biochemistry 20, 2671–2678.
[36] Wiskich, J.T. and Bonner, W.D. Jr. (1963) Plant Physiol. 38, 594–604.
[37] Drury, R.E., McCollum, J.P. and Garrison, S.A. (1968) Plant Physiol. 43, 248–254.
[38] Raison, J.K., Laties, G.G. and Crompton, M. (1973) Bioenergetics 4, 409–422.
[39] Diolez, P. and Moreau, F. (1985) Biochim. Biophys. Acta 806, 56–63.
[40] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[41] Chance, B. and Williams, G.R. (1955) J. Biol. Chem. 217, 383–393.
[42] Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177–182.
[43] Kamo, N., Muratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membr. Biol. 49, 105–121.
[44] Chernyak, B.V., Dukhovich, V.F. and Khodjaev, E. Yu. (1987) FEBS Lett. 215, 300–304.
[45] Valerio, M., Diolez, P. and Haraux, F. (1994) Eur. J. Biochem. 221, 1071–1078.
[46] Matsuno-Yagi, A. and Hatefi, Y. (1993) J. Biol. Chem. 268, 1539–1545.
[47] Schönknecht, G., Althoff, G., Apley, E., Wagner, R. and Junge, W. (1989) FEBS Lett. 258, 190–194.
[48] Valerio, M. and Haraux, F. (1993) FEBS Lett. 336, 83–86.
[49] Chernyak, B.V., Chernyak, V.Ya., Gladysheva, T.B., Kozhanova, Z.E. and Kozlov, I.A. (1981) Biochim. Biophys. Acta 635, 552–570.
[50] Valerio, M., De Kouchkovsky, Y. and Haraux, F. (1992) Biochemistry 31, 4239–4247.
[51] Vàzquez-Laslop, N. and Dreyfus, G. (1986) J. Biol. Chem. 261, 7807–7810.
[52] Glaser, E., Hamasur, B., Norling, B. and Andersson, B. (1987) FEBS Lett. 223, 304–308.
[53] Glaser, E., Hamasur, B., Tourikas, C., Norling, B. and Andersson, B. (1989) Plant Physiol. Biochem. 27, 471–481.
[54] Lötscher, H.-R., DeJong, C. and Capaldi, R.A. (1984) Biochemistry 23, 4140–4143.
[55] Paik, S.R., Jault, J.-M. and Allison, W.S. (1994) Biochemistry 33, 126–133.
[56] Di Pietro, A., Fellous, G., Godinot, C. and Gautheron, D.C. (1986) Biochim. Biophys. Acta 851, 283–294.
[57] Di Pietro, A., Penin, F., Godinot, C. and Gautheron, D.C. (1980) Biochemistry 19, 5671–5678.
[58] Murataliev, M.B. (1992) Biochemistry 31, 12885–12892.