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Activation and Deactivation of F_0F_1-ATPase in Yeast Mitochrondia

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The regulation of membrane-bound proton F_0F_1 ATPase by the protonmotive force and nucleotides was studied in yeast mitochondria. Activation occurred in whole mitochondria and the ATPase activity was measured just after disrupting the membranes with Triton X-100. Deactivation occurred either in whole mitochondria uncoupled with FCCP, or in disrupted membranes. No effect of Triton X-100 on the ATPase was observed, except a slow reactivation observed only in the absence of MgADP. Both AMPPNP and ATP increased the ATPase deactivation rate, thus indicating that occupancy of nucleotidic sites by ATP is more decisive than catalytic turnover for this process. ADP was found to stimulate the energy-dependent ATPase activation. ATPase deactivated at the same rate in uncoupled and disrupted mitochondria. This suggests that deactivation is not controlled by rebinding of some soluble factor, like IF1, but rather by the conversion of the F_1.IF1 complex into an inactive form.

KEY WORDS: Mitochondria, F_0F_1 ATPase, ATP synthase, ATP hydrolysis, IF1, Yeast, regulation, inactivation, proton gradient, detergent.

INTRODUCTION

F_0F_1[1] proton ATPase (or ATP synthase) is responsible for ATP synthesis in the inner membrane of mitochondria, the thylakoid membrane of chloroplasts and the cytoplasmic membrane of bacteria (review: Pedersen, 1996, and all papers in the same issue). The catalytic sector F_1, with subunits α, β, γ, δ and ε (stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$) is a water-soluble entity bearing catalytic and non-catalytic nucleotide binding sites (review: Boyer, 1993). The X-ray structure of the major part of this subcomplex has been elucidated in the case of the mammalian species (Abrahams et al., 1994; Bianchet et al., 1998). Isolated F_1 retains the ability to hydrolyze ATP. ATP hydrolysis probably results in the rotation of the γ subunit within the $\alpha_3\beta_3$ crown (Aggeler et al., 1995; Duncan et al., 1995; Sabbert et al., 1996; Noji et al., 1997). The F_0 sector is membranous and forms a proton-channelling device converting the energy of the electrochemical proton gradient $\Delta\mu_{H^+}$ into mechanical energy. It contains the three subunits a, b and c, like the E. coli enzyme (probable stoichiometry $a_1b_1c_{;9-12}$) and additional subunits specific to the mitochondrial enzyme (at least subunits 8, d, h, f and OSCP in yeast; Arselin et al., 1996). The whole F_0F_1 complex is thought to act as a rotatory proton-driven motor (reviews: Weber and Senior, 1997; Engelbrecht and Junge, 1997), the stator-

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[5] Abbreviations: F_0F_1, ATP-synthase; MF_0MF_1, mitochondrial ATP synthase; (M,C) F_1, catalytic sector of the (mitochondrial, chloroplast) ATP synthase; (M)F_0, membranous sector of the (mitochondrial) ATP synthase; IF1, natural inhibitory peptide; CCCP, carbonyl-cyanide m-chlorophenylhydrazone; FCCP, carbonyl-cyanide p-(trifluoromethoxy)phenylhydrazone; AMPPNP, 5′-adenylimidodiphosphate; $\Delta\mu_{H^+}$, transmembrane difference of electrochemical potential of protons; $\Delta\psi$, membrane potential; OSCP, oligomycin sensitivity conferring protein; Tris, tris(hydroxymethyl)aminomethane; Triton X-100, t-octylphenoxypolyethoxyethanol; PK, pyruvate kinase; LDH, lactate dehydrogenase, PEP, phosphoenolpyruvate; LDAO, lauryldimethylamine oxide.

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containing subunits a, b, OSCP, α and β and the rotor subunits c, γ, δ and ε in the case of the mitochondrial enzyme.

It has been known for many years that $\tilde{\Delta\mu}_{H^+}$ has an activatory effect on the F_0F_1 proton ATPase in addition to its protonmotive role (reviews: Schwerzmann and Pedersen, 1986; Harris and Das, 1991; Rouslin, 1991). This regulation involves an additional inhibitory subunit called IF1 (Pullman and Monroy, 1963). The size of IF1 varies between 60 and 80 aminoacids, dependent on the species. This inhibitory peptide is readily removed from the MF_0MF_1 complex, which gives an active ATPase. The binding site of IF1 is thought to be at the αβ interface (Mimura et al., 1993) and to involve the C-terminal domain of β following the DELSEED loop (Jackson and Harris, 1988). In yeast, two additional peptides have been proposed to be necessary to allow IF1 to exert its inhibitory effect (Hashimoto et al., 1983). The sequence of different IF1 peptides (from animals, yeast and plants) has been determined, and the minimal sequence(s) necessary for the inhibitory effect is under investigation (van Raaij et al., 1996; Lebowitz and Pedersen, 1996; Polgreen et al., 1995; Papa et al., 1996). $\Delta\mu_{H^+}$ is generally considered as favoring the release of IF1 from the core of the enzyme (Van de Stadt et al., 1973; Schwerzmann and Pedersen, 1981; Husain and Harris, 1983; Power et al., 1983; Klein and Vignais, 1983; Husain et al., 1985; Lippe et al., 1988a,b). However, some authors (Dreyfus et al., 1981; Beltran et al., 1984) proposed that the effect of $\Delta\mu_{H^+}$ could consist in shifting IF1 from an inhibitory to a non-inhibitory position. A docking site for IF1, distinct from the site responsible for the inhibition, was proposed in the case of beef heart mitochondria (Lopez-Mediavilla et al., 1993).

Nucleotides contribute to ATPase deactivation in the absence of $\Delta\mu_{H^+}$. It was early demonstrated (Pullmann and Monroy, 1963), and confirmed later (Gomez-Fernandez and Harris, 1978; Cintron et al., 1982) that MgATP was essential for ATPase inhibition by IF1. Lippe et al. (1988b) have shown that MgATP accelerates IF1 binding to membrane-bound MF_1, whereas Galante et al. (1981) have suggested that MgATP changes the MF_1-IF1 complex from a non-inhibited to an inhibited form. In plant mitochondria deenergized in the presence of MgATP, the inhibition of the ATP/ADP antiporter limited the ATPase deactivation, suggesting that enzyme turnover, more than ATP binding, governs this process (Chernyak et al., 1995). Without IF1, tightly bound ADP coming from the medium or from ATP hydrolysis inhibits the membrane-bound (Fitin et al., 1979) and the isolated (Di Pietro et al., 1986) enzyme. Taken together, and by analogy with CF_1 (Kleefeld et al., 1990), these data suggest that in the absence of IF1, inactivation could be actually produced by tightly bound ADP coming either from the medium or from ATP hydrolysis. In the presence of IF1, tightly bound ADP could originate only from ATP hydrolysis. However, the actual situation may be more complex.

Most studies on the role of IF1 and nucleotides in ATPase deactivation were carried out with inside-out submitochondrial particles. This approach has several advantages: i/ it allows the examination of the effects of IF1 addition, the study of the dissociation of radioactively labeled IF1, or the detection of IF1 by specific antobodies, ii/ it bypasses the nucleotide and phosphate carriers and then allows the concentration of nucleotides available for MF_1 to be controlled. Nevertheless, this approach tends to favor energy-dependent dissociation of IF1, because the aqueous volume available for IF1 release is considerably higher than the matrix space. It would be of interest to know what happens when MF_1 and IF_1 are confined within the matrix during the activation. In this report, we have studied the regulation of yeast MF_0MF_1 focusing on the deactivation process. We have adapted a method previously developed to study the ATPase regulation in plant mitochondria (Valerio et al., 1993; Valerio and Haraux, 1993; Chernyak et al., 1995). This approach consists of measuring ATP hydrolysis after fast disruption of the membranes of yeast mitochondria with a detergent. This allowed us to activate the ATPase with its F_1 moiety facing the endogenous medium contained in the matrix, and to study its deactivation in a medium of known composition. We were then able to clarify the role of nucleotides in activation and deactivation, and to make deductions about the IF1-F_1 interaction.

MATERIALS AND METHODS

Preparation, Storage and Use of Yeast Mitochondria

Cells of the diploid yeast strain 'Yeast Foam' were grown on a complete medium containing 2% lactate as carbon source (Arselin et al., 1976). Mitochondria were prepared from protoplasts as described by Guerin et al. (1979) and rapidly frozen in liquid nitrogen. Frozen mitochondria were kept at −80°C and could be used in functional experiments for more

than 1 year without alteration of their properties. Fresh mitochondria were used in experiments of IF1 immunodetection.

An aliquot was rapidly thawed before each set of experiments. Its activity then remained stable for at least one day. The respiratory activity (NADH oxidation[2]) was checked in the assay medium described below (pH 7.3, 25°C). The activities were: 230 to 280 natom O min ^{1} (mg protein)$^{-1}$ without nucleotide added, 1000 to 1400 natom O min^{-1} (mg protein)$^{-1}$ in State 3 (Chance and Williams, 1955), 210–340 natom O min^{-1} (mg protein)$^{-1}$ in State 4, 2100–2400 natom O min^{-1} (mg protein)$^{-1}$ in uncoupled conditions. The respiratory control (State 3/State 4 rates ratio) ranged between 3.3 and 5.3. The uncoupled/State 4 rates ratio ranged between 7.2 and 9.8. After the energisation of mitochondria by NADH and disruption of the membranes by Triton X-100 in the presence of MgATP, the initial rate of ATP hydrolysis ranged between 4.5 and 5.5 μmol ADP min^{-1} (mg protein)$^{-1}$.

Measurement of ATP Hydrolysis by pH Changes

Incubation and ATPase assays were carried out on the same 3 mL sample contained in the closed, stirred and thermostated (25°C) chamber of the apparatus previously described (Valerio et al., 1993). ATP hydrolysis was deduced from the production of scalar H^+ ions (Nishimura et al., 1962). pH variations were detected with a fast and sensitive glass electrode, and the suspension was titrated with HCl after each run, which allowed the conversion of pH changes into proton-equivalents and the time-response of the electrode to be checked. The activity of the respiratory chain was simultaneously controlled by O_2 consumption with the Clark-type electrode. The assay medium contained 0.65 M mannitol, 2 mM Tris-maleate, 3 mM potassium phosphate (pH 7.3), 1 mM $MgCl_2$, 1 mg/mL bovine serum albumin and mitochondria at a protein concentration of 0.05 mg/mL, unless otherwise indicated. Energization was initiated by adding 1 mM NADH. Three minutes later, the energisation step was terminated by adding Triton X-100, FCCP or antimycin. ATP hydrolysis was measured in the presence of 1 mM MgATP, added 1 min before collapsing the protonmotive force, or at different times later. Details of the different additions are given in the Results section or in the legends of the Figures. Addition of Triton X-100 or MgATP resulted in transient artifacts. These artifacts were revealed under conditions where no ATP hydrolysis occurred (absence of mitochondria, ATPase inhibited by oligomycin, or absence of ATP, depending on the cases). Their importance became negligible after 5 s. Therefore, the initial rate of hydrolysis was estimated between t = 5 s and t = 10 s.

Measurement of ATP Hydrolysis Using a ATP-Regenerating System

Mitochondria were energized in an incubation medium, then an aliquot was taken up and assayed for ATP hydrolysis in a reaction medium. The incubation medium, thermostated at 25°C, contained 0.65 M mannitol, 20 mM Tris-maleate, 3 mM potassium phosphate (pH 7.3), 1 mM $MgCl_2$, 17 mM KCl, 1 mg/mL bovine serum albumin, and mitochondria at aprotein concentration of 0.2 mg/mL.

In experiments on deactivation (Figs. 4–5), mitochondria were energized for 3 min in this medium by adding 2.5 mM NADH. Then, 120 μL of the suspension were taken up and immediately injected into the spectrophotometric micro-cuvette, stirred and thermostated at 25°C, and containing 1.08 mL of the reaction medium. The reaction medium (pH 7.3) was identical to the incubation medium, except that it was supplemented with 0.25 mM NADH (initial concentration), 2 mM phosphoenolpyruvate, 20 U/mL pyruvate kinase, 50 U/mL lactate dehydrogenase, and in some cases 5 μM AMPPNP. 15 s after the injection of the aliquot of energized mitochondria in the spectrophotometric cuvette, either FCCP (10 μM), or Triton X-100 (0.2%) was added, depending on the experiment. In the first case, MgATP (1 mM) and Triton X-100 (0.2%) were simultaneously added at given times (including zero time) after FCCP. In the second case, MgATP alone (1 mM) was added at different times (including zero time) after Triton X-100. In some cases, PK was initially omitted from the reaction medium (but phosphoenol pyruvate, pyruvate kinase and NADH were still present), to prevent ADP disappearance, and added only 5 s before starting ATP hydrolysis, to allow its spectrophotometric detection. In the absence of MgATP, no significant NADH oxidation occurred 5 s after Triton X-100 addition, showing that the destruction of the respiratory chain was rapid and complete. The initial absorbance decrease following ATP addition, due to some contaminating ADP, also terminated in less than 5 s. Therefore, the initial rate of ATP hydrolysis was measured between 5 s and 10 s after MgATP addition.

In experiments on activation (Fig. 7), mitochondria (0.2 mg/mL) were first injected into the incubation medium (25°C) in the presence of 2.5 μM FCCP and 2.5 μM MgATP (t = 0). Other additions (PEP, PK) are indicated in the Results section or in the legends of the Figures. In some experiments, 250 μM MgATP or 250 μM MgADP was added at t = 6.5 min. At t = 8 min, 0.5 mM NADH was added. A given time later, 120 μL of the suspension was injected into 1.08 mL of the reaction medium (25°C) supplemented with 1 mM MgATP and 0.2% Triton X-100. The initial rate of ATP hydrolysis was calculated as described above.

Measurements of the Membrane Potential $\Delta\psi$

Mitochondria (0.1 mg protein per ml) were injected into a cuvette placed in a fluorimeter and containing the incubation medium used in the experiments on reactivation, supplemented with 2.5 μM MgATP, 2.5 μM FCCP and 2 μM Rhodamine 123. The sample was stirred and thermostated at 25°C, and the fluorescence was continuously monitored (λ excitation = 485 nm, λ emission = 533 nm; Emaus et al., 1986). 6.5 min after injection of mitochondria, either MgADP (50 mM), or PEP (2 mM) + PK was added. 1 mM NADH was added 1.5 min later to generate the membrane potential.

Immunodetection of IF1

Freshly isolated mitochondria (0.6 mg mitochondrial protein) were incubated in 3 mL of assay medium (0.6 M mannitol, 10 mM Tris-maleate pH 6.0) at 25°C. After incubation with various effectors, 0.2% Triton X-100 was added, the sample was centrifuged at 100,000 g for 15 min at 4°C to remove insolubilized mitochondria. The supernatant was then spun at 300,000 g for 90 min at 4°C in a Beckman TL120 ultracentrifuge to pellet the enzyme. The latter was dissolved in 50 μL of dissociating buffer (Schagger and Von Jagow, 1987). Proteins of the supernatant were precipitated by 10% trichloroacetic acid addition. The pellet was washed twice with cold acetone, dried and solubilized in 50 μL of dissociating buffer. Ten μL aliquots were applied to a 16.5% polyacrylamide slab gel prepared according to Schagger and Von Jagow (1987). The slab gel was transferred on a Pro-Blott membrane (Applied Biosystems) that was probed with polyclonal antibodies raised against yeast IF1 at a dilution of (1/10,000). The blot was revealed with the ECL kit of Amersham. The intensity of IF1 in each lane was measured by densitometry. The intensities of IF1 in the pellet and the supernatant for each experiment were added and taken as 100%.

Reagents

All reagents were of analytical grade. ATP (containing 0.8% ADP) and ADP (containing 0.03% ATP) were purchased from Boehringer Mannheim.

RESULTS

Activated State of ATPase Revealed by Detergent Addition

A first set of experiments was carried out to define clear-cut conditions of measurement of the ATPase activity. NADH was first added to mitochondria to generate the protonmotive force ("energization" of the membrane) and consequently to activate the ATPase. After addition of MgATP, mitochondria were rapidly disrupted by injecting Triton X-100 in the sample. The disruption was quasi-instantaneous as visualized by turbidity changes (not shown). Fig. 1a shows typical pH-meter recordings, obtained upon addition of Triton X-100 to energized mitochondria in the presence of ATP, and converted into ATP equivalents (Nishimura et al., 1962). Curves 1 and 2 were obtained at two different protein concentrations. After a small artifact (less than 5 s duration), the initial slope gives the initial rate of ATP hydrolysis. The rate then decreases due to ATPase deactivation and to ADP accumulation. Since the oxidation of one NADH molecule in mitochondria results in the net consumption of one H^+ ion, we had to insure that any residual respiratory activity did not interfere with the pH-metric measurement of ATP hydrolysis. A control experiment (Fig. 1a, curve 3) showed that in the absence of added ATP, no significant pH variation could be detected 5 s after Triton X-100 addition. This indicates that the destruction of the respiratory chain was total and immediate, as confirmed by monitoring oxygen or NADH consumption (not shown). The detergent treatment thus allows the specific measurement of the rate of ATP hydrolysis with the pH-metric method (the present case, Figs. 1 and 2) as well as with the ATP-regenerating system catalyzing NADH oxidation (further experiments, Figs. 4, 5 and 7).

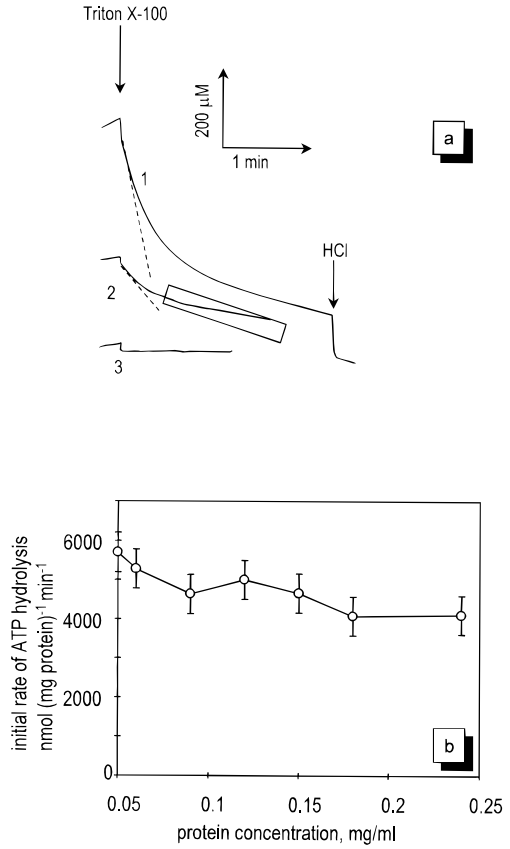

Fig. 1. pH-metric measurement of ATP hydrolysis upon addition of Triton X-100 to energized mitochondria. Conditions as described under Materials and Methods. a, pH-recording converted into ATP concentration. Additions are indicated by arrows. Triton concentration: 0.2%. Protein concentrations: curve 1, 0.25 mg mL^{-1}; curves 2 and 3, 0.05 mg mL^{-1}. Initial rates of ATP hydrolysis are evoked by dashed straightlines. Curves 1 and 2: mitochondria were preenergized for 4 min, with 1 mM MgATP added 1 min before Triton X-100. Curve 3: mitochondria were preenergized for 4 min, without MgATP addition. b, initial rate of ATP hydrolysis as a function of protein concentration.

Fig. 1b shows that the specific ATPase activity, as estimated by the initial rates, slightly decreased with the mitochondrial protein concentration. It was found to be practically independent of the concentration of Triton X-100 (data not shown). In the following, low concentrations of proteins (0.05 to 0.06 mg mL^{-1}) and 0.2 % Triton X-100 were used.

Deactivation of ATPase in Intact Yeast Mitochondria

We have invesigated the rate of deactivation of ATPase in intact mitochondria. This was achieved i) by adding FCCP to pre-energized mitochondria in order to collapse the protonmotive force, and ii) by adding Triton X-100 at a range of various times in order to start the measurement of ATP hydrolysis[3]. In some experiments, MgATP (1 mM) was present during the time separating FCCP and Triton X-100 additions. In other experiments, MgATP was added together with Triton X-100. The time-course of the experiment is depicted in the diagram shown at the top part of Fig. 2 (condition a). Fig. 2a shows the rate of ATP hydrolysis, measured immediately after the injection of Triton X-100, as a function of the time separating FCCP and Triton X-100 additions. The ATPase activity clearly decreased with time in the presence of FCCP. The decay was fast when 1 mM MgATP was present from the beginning (closed circles), and relatively slow when MgATP was only added at the same time as Triton X-100 (open circles) to reveal the ATPase activity. This difference shows that MgATP accelerates the deactivation.

In Fig. 2b, a similar experiment was done, but instead of collapsing the protonmotive force with FCCP, we stopped its generation by adding 2 µM antimycin, which preserved the coupling properties of the inner mitochondrial membrane. The ATPase deactivated rapidly in the absence of MgATP (open circles) but not in its presence (closed circles). In the latter case, the protonmotive force generated by ATP hydrolysis was clearly sufficient to keep MF_0MF_1 in its activated form for a long time. These data show that in yeast mitochondria, a protonmotive force, produced either by respiration or by ATP hydrolysis, is necessary to maintain the ATPase activity.

Reactivatory Effect of Triton X-100

The ATPase deactivates not only in intact mitochondria, but also in disrupted mitochondria. Indeed, in the kinetics of ATP hydrolysis displayed in Fig. 1a, the rates progressively decreased after Triton X-100 addition, as expected from the absence of the protonmotive force due to the destruction of the membranes. This behavior is confirmed in the progression curves displayed in Fig. 3a (two kinetics, with different initial levels of activity, are shown). However, due to the presence of detergent, the situation is somewhat complex. Fig. 3b indeed shows progression curves of ATP hydrolysis monitored not by pH-metry but by the ADP-dependent NADH oxidation catalyzed by the regenerating PK-LDH system. The pattern was quite different from the pH measurements. With preen-

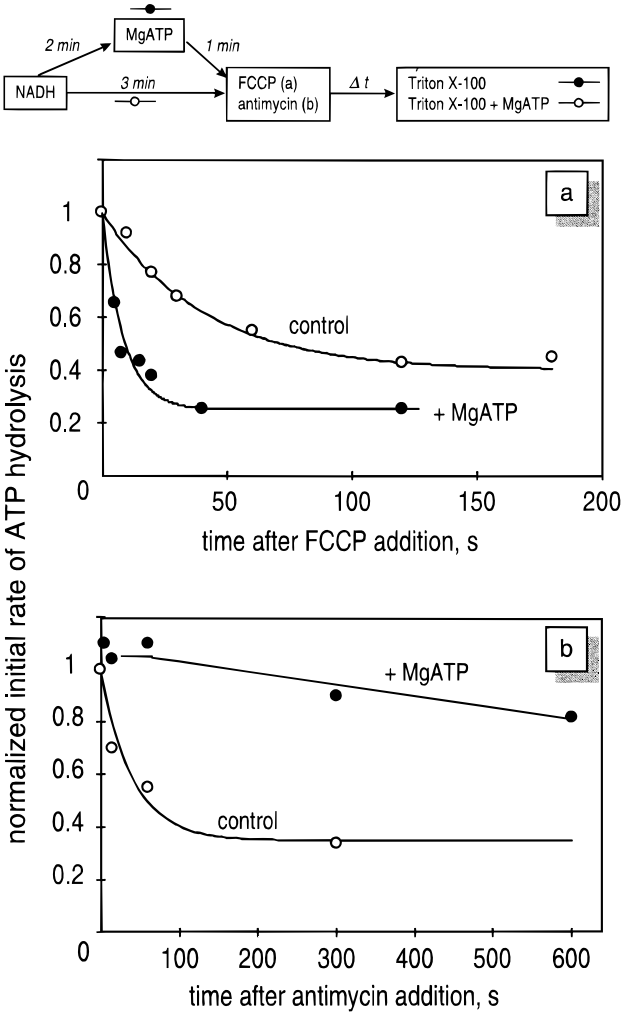

Fig. 2. Initial rates of ATP hydrolysis at different times after collapsing the protonmotive force. Conditions as described under Materials and Methods, pH-metric measurement. The sequence of additions corresponding to the different experiments is indicated above the plots. The initial rate of ATP hydrolysis was measured after the last addition. Triton X-100 concentration: 0. 2%. Protein concentration, 0.05 mg mL^{-1}. Open circles, 1 mM MgATP was added together with Triton X-100; closed circles, 1 mM MgATP was added 1 min before Triton X-100. a, rate of ATP hydrolysis as a function of the time between FCCP (10 μM) and Triton X-100 additions. b, rate of ATP hydrolysis as a function of the time between antimycin (2 μM) and Triton X-100 additions. Rates are normalized to that obtained by adding Triton X-100 at the same time as FCCP (a) or antimycin (b).

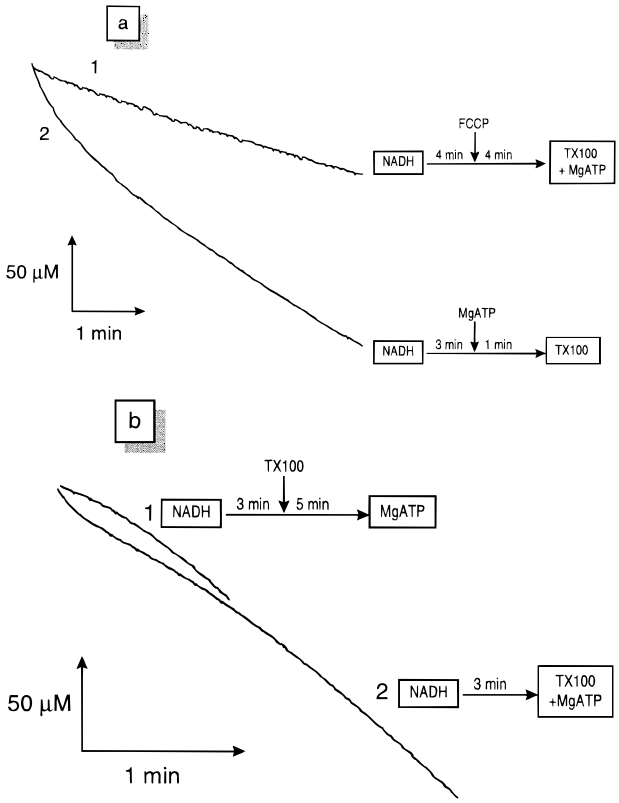

Fig. 3. Progression curves of ATP hydrolysis upon Triton X-100 addition. Conditions as described under Materials and Methods. The sequence of additions corresponding to the four different experiments is indicated near the traces, the measurement starting after the last addition. Triton X-100 concentration: 0. 2%. a, pH-metric measurement. Protein concentration, 0.05 mg mL^{-1}. Curve 1, mitochondria were deenergized with 10 μM FCCP for 4 min, and 1 mM ATP was added at the same time as Triton-X-100. Curve 2, mitochondria were energized for 4 min with 1 mM NADH, and 1 mM ATP was added 1 min before Triton X-100. b, spectrophotometric measurement by NADH oxidation. Protein concentration in the reaction medium, 0.02 mg mL^{-1}. Curve 1, mitochondria were energized for 3 min, and then disrupted with Triton X-100; 1 mM MgATP was added 5 min later. Curve 2, mitochondria energized for 3 min, then disrupted with Triton X-100 added at the same time as 1 mM MgATP.

ergized mitochondria (Fig.3b, curve 2), the rate of ATP hydrolysis after Triton X-100 addition was initially high, then rapidly decreased, and finally increased to reach a stationary regime. With mitochondria disrupted with Triton X-100 5 min before ATP addition (Fig.3b, curve 1), the activity was initially low, as expected from the disappearence of the protonmotive force, but then progressively increased, to reach the same final level as in curve 2. In contrast with the pH-metric experiments, it appeared here that a reactivation process, likely due to Triton X-100, was superimposed to the normal deactivation process. The main difference between these two experimental conditions lies in the fact that in the case of the spectrophotometric detection, ATP is continuously regenerated by the enzymatic system, avoiding ADP accumulation. These crossed effects of Triton X-100 and nucleotides were studied

in more detail so they can be taken into account in the deactivation study.

Deactivation and Reactivation of MF_0MF_1 in the Presence of Triton X-100 and Effect of Nucleotides

We have then investigated more in depth the deactivation of ATPase in mitochondria disrupted with Triton X-100, its reactivation by this detergent, and the possible role of nucleotides in these two processes. All the experiments were done using the spectrophotometric assay for ATP hydrolysis (the PK-LDH system). Pre-energized mitochondria were disrupted with Triton X-100 in different conditions, and ATP hydrolysis was initiated by adding MgATP at different times after Triton X-100 addition. Unless otherwise stated, the reaction medium contained PK, PEP, LDH and NADH from the beginning. A scheme of the procedure and the results of the experiments are displayed in Fig. 4.

The plot shows the initial rate of ATP hydrolysis as a function of the time separating Triton X-100 and MgATP additions. In the first condition (control, open circles), the activity decreased in two minutes down to about 50% of its starting value and then rose to reach approximately 70% of this value after 10 minutes. This behavior resembles that observed when ATP hydrolysis was continuously measured in the presence of Triton X-100 (see Fig. 3b), except that deactivation and reactivation were slower. For comparison, the time-dependency of the instantaneous rate of ATP hydrolysis in the presence of Triton X-100 is presented in Fig. 4 (thick continuous curve). This plot is the first derivative of the typical spectrophotometric recording, e.g. Fig. 3b, curve 2. This comparison strongly suggests that deactivation and Triton X-100-induced reactivation are controlled by ATP, added at a high concentration (thick continuous curve), or initially present in mitochondria and diluted into the reaction medium after Triton X-100 addition (open circles).

To check this hypothesis, we repeated the experiment of Fig. 4 with the following change: PK was omitted from the initial reaction medium (which however contained PEP, LDH and NADH) but it was added only 5 seconds before the addition of MgATP in order to allow the measurement of ATP hydrolysis. In this case (Fig. 4, triangles), most of the deactivation following Triton X-100 addition occurred in the absence of regeneration of ATP. The initial decay of ATPase activity was similar to that observed in the presence of the

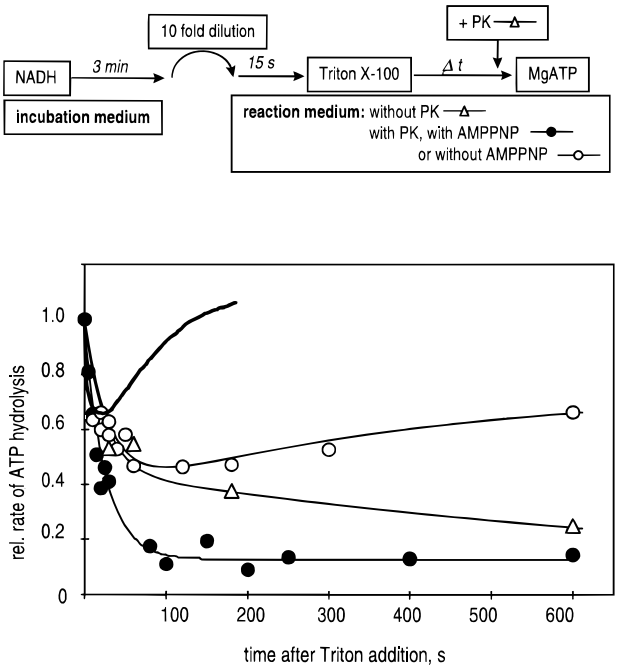

Fig. 4. Rate of ATP hydrolysis as a function of time after Triton X-100 addition. Conditions as described under Materials and Methods. The procedure corresponding to the different experiments is indicated above the plots. The rate of ATP hydrolysis was measured after the injection of MgATP into the reaction medium. Continuous thick curve: instantaneous rate after simultaneous injection of Triton X-100 and 1 mM MgATP, obtained by taking the first derivative of the spectrophotometric recording. Other plots: initial rates of ATP hydrolysis. Open circles, no AMPPNP addition, PK was initially present in the reaction medium. Open triangles, no addition, PK was omitted in the reaction medium and added 5 s before MgATP. Closed circles, 5 μM AMPPNP was initially present in the reaction medium. Rates are normalized to that obtained by adding MgATP at the same time as Triton X-100.

regenerating system. However, the long-term reactivation disappeared, presumably because the endogenous ATP was transformed into ADP. This is consistent with the previous observation (Fig. 3) that Triton X-100-induced reactivation was not observed when the ATPase activity was monitored by pH-metry, i.e. without regenerating ATP.

In a third experiment (Fig. 4, closed circles), we have studied the deactivation of ATPase in a medium supplemented with 5 μM AMPPNP. This concentration was found to be sufficient to compete with endogenous nucleotides during the deactivation, but not with ATP added to promote ATP hydrolysis. The decay of activity was faster (50% of the activity was lost in 15 s) and more complete (the residual activity was only 15%) than those observed in the presence of endogenous nucleotides. No reactivation was observed. These

experiments revealed than in disrupted mitochondria, deactivation of ATPase and its reactivation by Triton X-100 depend on the presence of nucleotides, endogenous or added.

Deactivation of MF_0MF_1 and Effect of Nucleotides in Intact Mitochondria Uncoupled by FCCP

We have also examined the effects of nucleotides on the deactivation of ATPase in intact mitochiondria in the presence of FCCP. In the experiments depicted in Fig. 5, preenergized mitochondria were rapidly deenergized by addition of FCCP 10 μM, then Triton X-100 and MgATP were simultaneously injected into the reaction medium. The different nucleotide conditions parallelled those of Fig. 4. The plot of Fig. 5 shows the initial rate of ATP hydrolysis as a function of the time separating FCCP and MgATP + Triton X-100 additions. Deactivation was never complete, probably due to the existence of a residual protonmotive force (FCCP cannot be used at too high concentrations to avoid inhibitory effects on the ATPase). Without addition of nucleotides (that is with endogenous nucleotides) the decay of activity was the same with (open circles) or without (open triangles) PK during the deactivation. In the presence of AMPPNP 5 μM, the rate and the extent of deactivation were increased. The effect of AMPPNP in intact mitochondria proves that this ATP analogue readily crosses the inner mitochondrial membrane, probably via the ATP/ADP antiporter. The general behavior then looks like that observed when deactivation occurred in the presence of Triton X-100 (Fig. 4), except that the slow reactivation was not observed here.

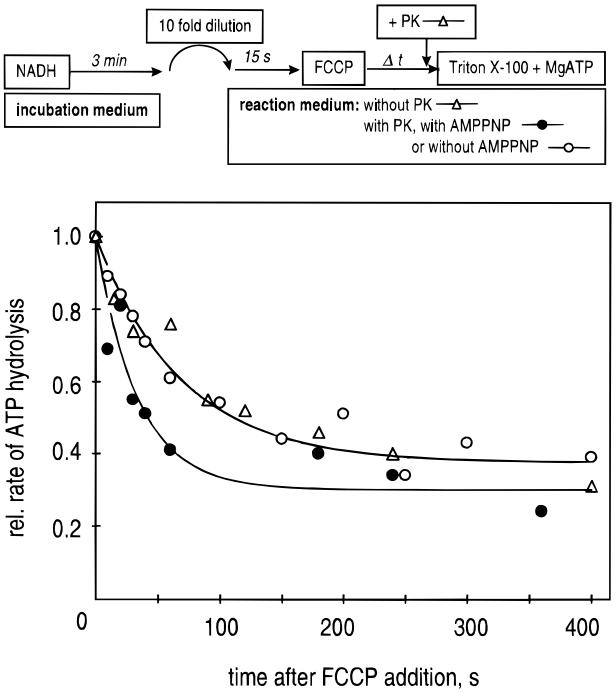

Fig. 5. Rate of ATP hydrolysis as a function of time after FCCP addition. Conditions as described under Materials and Methods. The procedure corresponding to the different experiments is indicated above the plots. The rate of ATP hydrolysis was measured after the simultaneous injection of Triton X-100 and MgATP into the reaction medium. Open circles, no addition, PK was initially present in the reaction medium. Open triangles, no AMPPNP addition, PK was omitted in the reaction medium and added 5 s before MgATP. Closed circles, 5 μM AMPPNP was initially present in the reaction medium. Rates are normalized to that obtained by adding MgATP + Triton X-100 at the same time as FCCP.

Energy-Dependent Release of IF1

We have looked if energization of mitochondria led to the release of the inhibitory peptide IF1. The experiment consisted of incubating fresh mitochondria at pH 6 at room temperature under given conditions, solubilizing the MF_0MF_1 complex by Triton X-100, separating them from the medium by ultracentrifugation, and detecting IF1 by Western blot analyses of enzyme pellets and supernatants. Fig. 6 presents the immunoblots obtained under the different conditions. In one case (deenergized conditions), mitochondria were incubated with 6 μM CCCP for 30 min before Triton X-100 addition, 100 μM AMPPNP and 20 μg oligomycin/mg protein being added 5 min before Triton X-100. About the same amount of IF1 (42% and 58%) was detected in the enzyme pellet and in the medium, respectively. In the so-called 'energized conditions', mitochondria were incubated with 2 mM NADH 4 min before Triton X-100 addition. In this case, 82% of IF1 was detected in the medium. This shows that energization favors the release of IF1 from the ATP synthase.

Energy-Dependent Activation of MF_0MF_1 in the Absence or in the Presence of ADP

Since nucleotides play a role in the deactivation of ATPase in the absence of protonmotive force, one may expect that they also interfere with the process

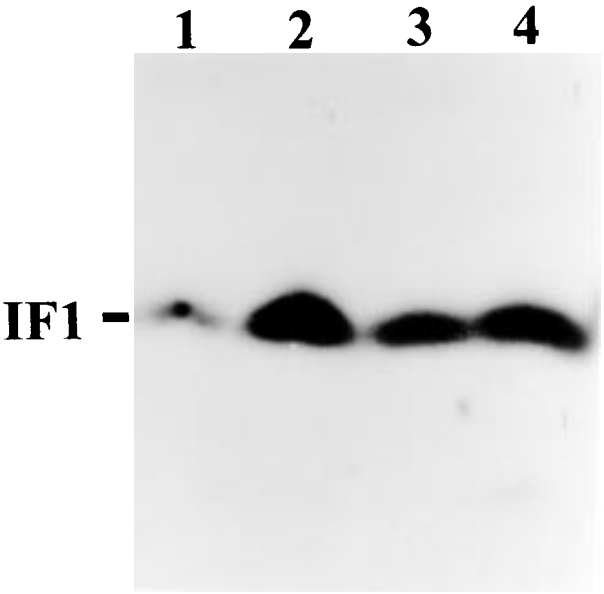

Fig. 6. Immunodetection of IF1 in energized and deenergized conditions. Conditions as described under Materials and Methods. Lanes 1 and 3, 300,000 g pellets of Triton X-100-treated mitochondria incubated either with 2 mM NADH (energized conditions) or with 6 μM CCCP, 100 μM AMPPNP and 20 μg of oligomycin / mg protein (deenergized conditions), respectively. Lanes 2 and 4, 300,000 g supernatant of Triton X-100-treated mitochondria incubated either with NADH (energized conditions) or with 6 μM CCCP, 100 μM AMPPNP and 20 μg oligomycin / mg protein (deenergized conditions), respectively. Samples were submitted to a Western blot analysis. Blots were revealed with antisera raised against IF1.

of energy-induced activation. We have then studied the activation of the ATPase by the protonmotive force and the sensitivity of this process to nucleotides. Mitochondria were first incubated with 2.5 μM FCCP and 2.5 μM MgATP for 8 min to fully deactivate the ATPase, and then supplemented with 0.5 mM NADH. At a given time after the addition of NADH (from 0 to 60 s), an aliquot was put into the reaction medium containing ATP, Triton X-100 and the regenerating system, and ATP hydrolysis was monitored.

Figure 7 shows the initial rate of ATP hydrolysis as a function of the time of reactivation in the incubation medium under different conditions. Despite the presence of 2.5 μM FCCP, the protonmotive force remained high enough to allow a significant reactivation of ATPase. In Fig. 7a, closed circles represent the activities obtained when 250 μM ADP were present during reactivation, and open circles represent the activities obtained when reactivation occurred in the absence of added nucleotides but in the presence of PK + PEP in the incubation medium to eliminate any endogenous ADP. The rate and extent of ATPase activation were clearly diminished in the second case.

Since PEP can bind magnesium, which could have a indirect effect on ATPase activation, we have verified that PEP without PK in the incubation medium had no effect on the kinetics of activation (not shown). Therefore, decrease of ATPase activation is directly related to the disappearance of ADP and/or the appearance of ATP, and is not related to magnesium removal.

Fig. 7b compares the reactivation in the presence of 250 μM ADP (closed circles) and 250 μM ATP (closed squares). It can be seen that ATP failed to stimulate the activation of ATPase to the same extent as ADP. Moreover, this stimulation was fully abolished in the presence of PK + PEP (open squares), which shows that the partial stimulation observed with ATP alone was actually due to some ADP. HPLC measurement showed that ATP contained about 0.8% ADP.

The effect of ADP on the $\Delta\tilde{\mu}_{H^+}$-dependent activation of ATPase could be either a direct effect on the enzyme or due to some increase of the protonmotive force. First, we have verified that the maximal capacity of the respiratory chain (in the presence of 10 μM FCCP) was not affected by the nucleotide conditions, which makes improbable an effect of nucleotides on the generation of the protonmotive force. Second, we monitored the generation of $\Delta\tilde{\mu}_{H^+}$ in mitochondria partially uncoupled by 2.5 μM FCCP, by using the fluorescence of Rhodamine 123, which is sensitive to the membrane potential (Emaus et al., 1986; the chemical component of $\Delta\tilde{\mu}_{H^+}$ is negligible here).

Fig. 8 shows the effect of 250 μM ADP on the generation of $\Delta\psi$ upon a NADH addition under conditions analogues to those of Fig. 7. The initial rise of $\Delta\psi$ was the same either in the presence of 250 μM MgADP or in the presence of PEP + PK (that is 2.5 μM MgATP), which is consistent with the identical functioning of the respiratory chain. The maximum $\Delta\psi$ reached in the presence of ADP was somewhat lower than in its absence, which suggests that the phosphorylating proton flux significantly contributes to the dissipation of the protonmotive force, even in partially uncoupled conditions. This was confirmed by the fact that the time required to oxidize all added NADH (1 mM) was slightly lower with ADP (7.5 min) than without ADP

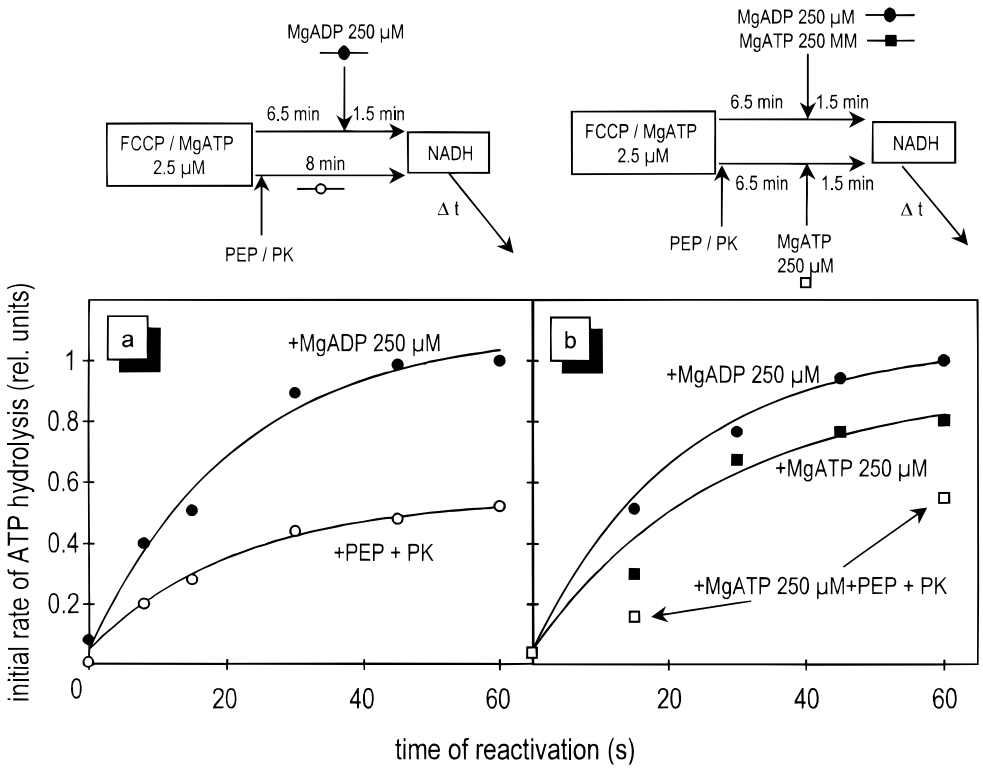

Fig. 7. Rate of ATP hydrolysis as a function of time after NADH addition to deenergized mitochondria. Conditions as described under Materials and Methods. The procedure corresponding to the different experiments is indicated above the plots. After the Δt reactivation time, mitochondria were diluted into the reaction medium contained in the spectrophotometric cuvette and the initial rate of ATP hydrolysis was measured. a, reactivation in the presence of 250 µM ADP (-●-) or in the presence of PK and PEP (-○-). b, reactivation in the presence of 250 µM ADP (-●-), 250 µM ATP (-■-), or 250 µM ATP, PK and PEP (-□-).

(8 min). Whatever it may be, we can conclude that the stimulation of ATPase activation by ADP cannot be attributed to a higher protonmotive force and that it is due to a direct effect on the enzyme.

DISCUSSION

Effect of Triton X-100 on the Stability of the F_1-IF1 Complex

In our conditions, the effect of Triton X-100 essentially consists in disrupting the mitochondrial membranes, without significant side effect on the ATPsynthase complex. This contrasts with the effects of some other detergents, such as LDAO, used in previous studies on plant mitochondria. LDAO was found to greatly stimulate the ATPase activity, to trap the activated ATPase in its active state, and to instantaneously make the ATPase insensitive to inhibitors of the F_0 sector, which was interpreted as an internal uncoupling of the F_0F_1 complex (Valerio et al., 1993; Chernyak et al., 1995). In yeast mitochondria, we found that LDAO also trapped the active state of ATPase but, in contrast with that was observed in plant mitochondria, this detergent had an inhibitory effect on ATPase (not shown). No such effects were observed with Triton X-100.

The only effect on the ATPase common to LDAO and Triton X-100 is the ability to slowly reactivate the deactivated ATPase (Chernyak et al., 1993; this report, Figs. 3b and 4). This reactivation was considerably accelerated by addition of 1 mM ATP, but prevented by AMPPNP. ADP prevents the detergent-induced activation. This effect of Triton X-100 is probably due to the irreversible release of IF1, the binding of which to the core of MF_1 being more or less tight, depending on the nucleotide occupancy. Since, as discussed below, the enzyme activated by the protonmotive force likely contains IF1 bound in a non-inhibitory way, the nucleotide-dependent release of IF1 by Triton X-100 probably exists for the activated as well as for the deactivated forms of MF_0MF_1.

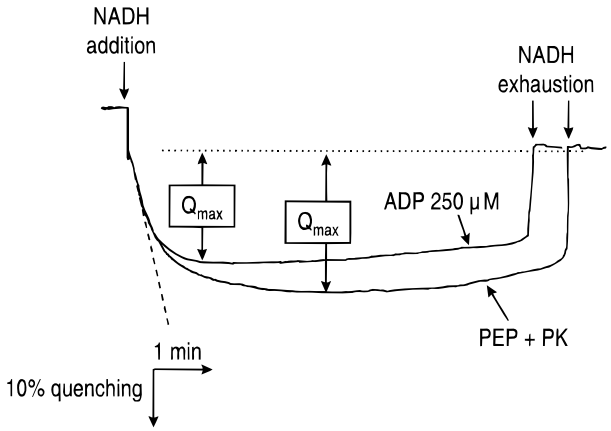

Fig. 8. Time-course of Rhodamine 123 fluorescence quenching after NADH addition to partially uncoupled mitochondria. Effect of ADP. Conditions as described under Materials and Methods. The initial fast decrease of fluorescence upon addition of 1 mM NADH is due to a direct energy-independent quenching. The fluorescence level in resting state is represented by a horizontal dotted line. This level is rapidly recovered after NADH exhaustion. The initial rate of fluorescence quenching is evoked by a dashed line; its value was 36.3 ± 3.2% min^{-1} in the presence of 250 µM MgADP, and 36.8 ± 3.1% min^{-1} in the presence of 2 mM PEP and PK. Q_{max}, maximal magnitude of the fluorescence quenching; its value was 20.5 ± 2.5% in the presence of 250 µM MgADP, and 26.3 ± 0.6% in the presence of 2 mM PEP and PK. The time required to oxidize all the NADH, as defined by the interval between the two vertical arrows, was 7.5 ± 0.7 min in the presence of 250 µM MgADP, and 8.0 ± 0.5 min in the presence of 2 mM PEP and PK (averaged values from four independent experiments).

Although this effect of detergent might reveal some interesting features of the MF_1-IF1 interaction (dependency on nucleotides, catalytic turnover, etc.), it seemed us too complex to deserve, at the present time, a detailed study, and we preferred to focus on the deactivation process. The reactivatory effect of the detergent may however be of practical interest. By identifying the Triton X-100-induced activity (final slope in Fig. 3b) to that of all the MF_0MF_1 population, we are able to express the energy-dependent activity (initial slope in Fig. 3b) as an activated fraction of the enzymes. We thus found that after some minutes of incubation of frozen-thawed mitochondria with NADH, 70% to 90% of the ATPases were activated by the protonmotive force.

Role of nucleotides and of the catalytic turnover in the ATPase deactivation

Although ATP has been known for a long time to favor IF1 inhibition of ATPase, it is still difficult to know if this effect is simply due to the occupancy of some site(s) by ATP or to the existence of a microscopic state transiently formed during the catalytic turnover. Previous experiments made on plant mitochondria suggested that the catalytic turnover played an important role in the deactivation in the absence of protonmotive force (Chernyak et al., 1993). For example, inhibition of the ATP/ADP antiporter by carboxyatractyloside was found to slow down the decay of ATPase activity in de-energized mitochondria, suggesting that continuous feeding with the substrate was necessary for a maximal rate of deactivation. However, from the present study, it is clear that AMPPNP also accelerates the deactivation of ATPase, in the same way as does ATP. This demonstrates that occupancy of nucleotidic site(s) by ATP, or by its non-hydrolysable analogue, significantly contributes in itself to the process of deactivation.

Role of IF1

Many reports have mentioned not only the energy-dependent dissociation of IF1 but also its rebinding under deenergized conditions. In bovine heart submitochondrial particles, a good kinetic correlation was found between the release of IF1 and the increase of ATPase activity (Lippe et al., 1988a). However, the use of submitochondrial particles is not devoid of side effects, and it is possible that exposure of the F_1,IF1 complex on the outside of these vesicles favors its dissociation. In the present report, we could detect a difference in binding of IF1 to mitochondrial membranes according to the energetic state of mitochondria (Fig. 6). When mitochondria were deenergized in the presence of AMPPNP before detergent treatment, approximately half of IF1 was found to be bound to the membranes. It is possible that mitochondria contain more than one IF1 per MF_1 molecule, which could explain why IF1 was not found totally bound. When mitochondria were preenergized before detergent treatment, more IF1 was found in the medium and less IF1 was found associated to the ATP synthase. At first sight, this would corroborate the energy-dependent release of IF1. However, we know that in the presence of endogenous ATP, Triton X-100 may slowly reactivate the ATPase, and it is possible that the protonmotive force only weakens the interaction between F_1 and IF1, allowing the detergent to slowly extract IF1.

An argument against the energy-dependent release of IF1 in mitochondrial matrix comes from the

comparison of the data of Figs. 4 and 5. In one case (Fig. 4), the deactivation was followed after disrupting the membranes with Triton X-100. In the other case (Fig. 5), the deactivation was followed after uncoupling the membranes with FCCP. We can see that within the first tens of seconds, the rate of ATPase deactivation is quite similar in both conditions. This is not expected in the case of rebinding of previously dissociated IF1 or of any soluble factor, because disruption of the membrane mitochondria by a detergent immediately leads to the high dilution of compounds of the matrix into a large volume. This should considerably slow down IF1 rebinding, and then ATPase deactivation. Since this was not observed, we conclude that IF1 was probably already bound in a non-inhibitory way, and that the decay of activity with time is likely related to the change of position of bound IF_1. This change would be accelerated by ATP or by AMPPNP.

Symmetrical Roles of ATP and ADP in the Regulation of MF_0MF_1

We have shown that the binding of ATP to MF_1 accelerated its deactivation in the absence of a protonmotive force, probably by acting on a previously formed F_1-IF1 complex. We have also studied the reactivation of ATPase by the protonmotive force and found that reactivation was faster and more extensive in the presence of ADP than in the presence of ATP (Fig. 7). Since it was not possible to eliminate the nucleotides (it was only possible to convert ADP into ATP), it is not easy to decide a priori if reactivation is stimulated by ADP or just inhibited by ATP. In fact, our experiments suggest that ADP does have a stimulatory effect. Indeed, in Fig. 7c, the reactivation in presence of 250 μM ATP (containing about 2 μM ADP) was significantly altered when this contaminating ADP was converted into ATP by PK. Since the relative variation of the ATP concentration was negligible, the change was due to ADP. This indicates that ADP, at a low concentration, stimulates the energy-dependent activation. This conclusion differs from that of Lippe et al. (1988b), who found that ATP alone regulated the energy-dependent activation of ATPase in bovine heart submitochondrial particles.

Rate of ATPase Activation

In partially uncoupled mitochondria, the activation of ATPase requires about 1 min (Fig. 7). Fig. 8 shows that 1 min is approximately the time needed to reach, in these conditions, the steady state $\Delta\psi$ as monitored by Rhodamine 123 fluorescence. Although the relationship between the fluorescence quenching and the amount of active enzyme at equilibrium is a priori not simple, it can be proposed that in the curves displayed in Fig. 7, the rise of activity is kinetically limited by the rise of the protonmotive force, and not by the conversion of the inactive form of the enzyme to the inactive one, which would be very fast in itself.

Conclusion

Activation and deactivation of ATPase can be studied in submitochondrial particles or in whole mitochondria. The present approach, consisting of rapid disruption of mitochondria by a detergent, seems to present some advantages, one of them being to avoid the exposure of the F_1,IF1 complex to a large external volume during the activation. At the present time, we are not able to know whether the discrepancies between our data and some of the results of the literature are due to the different experimental approaches or to the different sources (animal or yeast) of mitochondria. We would like to stress that both approaches are necessary and probably complementary to study this very complex process.

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