Interaction of Mg^{2+} with $\text{F}_0 \cdot \text{F}_1$ mitochondrial ATPase as related to its slow active/inactive transition

Vladimir V. BULYGIN and Andrei D. VINOGRADOV*

Department of Biochemistry, School of Biology, Moscow State University, Moscow 119899, U.S.S.R.

Bovine heart submitochondrial particles incubated with a low concentration of ADP in the presence of Mg^{2+} and passed through a Sephadex column equilibrated with EDTA exhibit sensitivity of their initial ATPase activity to preincubation with Mg^{2+} . By using particles thus prepared, several characteristics of a Mg^{2+} -specific inhibitory site on $\text{F}_0 \cdot \text{F}_1$ ATPase were studied. The inhibition was shown to be both time- and Mg^{2+} -concentration-dependent, with an equilibrium constant (at infinite time) of $2 \times 10^{-6} \text{ M}$ (25 °C, pH 7.5). The dependence of the pseudo-first-order rate constant for the inhibition process on Mg^{2+} concentration suggests the presence of a single Mg^{2+} -binding site with $K_s = 1.1 \times 10^{-4} \text{ M}$. The data obtained are consistent with a two-step mechanism of Mg^{2+} - $\text{F}_0 \cdot \text{F}_1$ interaction which results in a loss of the ATPase activity; it includes rapid pH-dependent binding of Mg^{2+} at the site with $K_s = 1.1 \times 10^{-4} \text{ M}$, followed by a slow interconversion of the Mg^{2+} - F_1 complex into inactive ATPase ($k_{\text{in}} = 0.65 \text{ min}^{-1}$, $k_{\text{act}} = 0.01 \text{ min}^{-1}$). The Mg^{2+} -inhibited ATPase is very slowly ($t_{1/2} \sim 90 \text{ min}$) re-activated in the presence of EDTA. The rate of EDTA-induced re-activation is pH-independent and can be dramatically increased by added ATP, P_i and sulphite. The dissociation constants for free ATP and P_i ($5 \times 10^{-7} \text{ M}$ and $1 \times 10^{-3} \text{ M}$ respectively) and the maximal activation rates were determined by measuring the hyperbolic dependencies of the EDTA-induced re-activation of Mg^{2+} -de-activated ATPase on the concentrations of the accelerating ligands. Taken together, the data obtained show two functionally detectable free nucleotide-specific binding sites, one site for P_i and one Mg^{2+} -specific ATPase-inhibitory site on the $\text{F}_0 \cdot \text{F}_1$ mitochondrial ATP synthase complex.

INTRODUCTION

$\text{F}_0 \cdot \text{F}_1$ -ATPase (ATP synthase) is an oligomeric membrane-bound enzyme which couples proton translocation with ATP hydrolysis (synthesis) in the membranes of mitochondria, chloroplasts and bacteria. The structural, catalytic and regulatory properties of the enzyme from different sources have been extensively reviewed [1–5], and the remarkable similarity of their subunit arrangement, the number and properties of nucleotide-binding sites and the sensitivity of ATP hydrolysis catalysed by the F_1 component to inhibitors and activators have become evident. Several hypothetical mechanisms for ATP synthesis (hydrolysis) coupled with vectorial proton flow through the F_0 component have been proposed [6–8]. None of them, however, is conclusively proved.

An intriguing feature of the enzyme which has been demonstrated both for purified soluble F_1 species and for the membrane-bound $\text{F}_0 \cdot \text{F}_1$ is a slow active/inactive transition of their ATPase activity, which is under a complex control of adenine nucleotides, P_i and Mg^{2+} (see [9] and references cited therein). In 1975, Moyle & Mitchell [10] showed that preincubation of rat liver submitochondrial particles or the soluble F_1 from bovine heart mitochondria in the presence of Mg^{2+} results in a slow (as compared with the enzyme turnover) de-activation of their ATPase activity. De-activated ATPase was shown to be susceptible to anion-induced activation which occurs on a time scale of minutes [10]. It soon became evident that ADP bound at some high-affinity nucleotide-specific site of F_1 is a prerequisite for the Mg^{2+} -induced de-activation and anion-induced activation of ATPase [11–14]. Accumulating evidence suggests that the phenomenon which can equally be termed as ADP-induced Mg^{2+} -dependent, or Mg^{2+} -induced ADP-dependent, slow de-activation of F_1 -type ATPases and their subsequent activation is responsible for the light-dependence of ATP

synthesis/hydrolysis in chloroplasts [15–17] and for the hysteretic behaviour of mitochondrial ATPase [18–21]. Studies on ADP (Mg^{2+})-dependent de-activation and ATP (Mg^{2+})-dependent activation of mitochondrial ATPase revealed that the well-known inhibitory effect of azide and stimulating effect on sulphite are also due to the slow active/inactive transition primarily controlled by the ATP/ADP ratio [14]. F_1 -ATPase contains a total of six adenine-nucleotide-binding sites [22]; three sites exchange bound nucleotides during ATP hydrolysis [22]. The isolated F_1 component has been shown to contain a single tightly bound Mg^{2+} [23], in addition to three sites where Mg^{2+} can be bound together with ATP at the exchangeable nucleotide-binding sites [24, 25] and one site responsible for the Mg^{2+} -induced F_1 in-activation accompanied by dissociation of the enzyme [26]. The latter site was not detected by an inhibitory effect of Mg^{2+} on the intact $\text{F}_0 \cdot \text{F}_1$ -ATP synthase complex [26]. Clearly, the presence of three 'exchangeable' nucleotide-binding sites, in addition to at least one P_i -binding site [27] and one or possibly two Mg^{2+} -specific site(s) [23,24] per F_1 molecule, gives numerous possible enzyme-substrate (product) complexes which may exist during the steady-state operation of the enzyme in either direction. Surprisingly, simple Michaelis-Menten kinetics for ATP hydrolysis [13] were observed under the conditions where the hysteretic enzyme behaviour was excluded. Whether ADP tightly bound to F_1 in the presence of Mg^{2+} is located at the enzyme catalytic site or at some regulatory site(s) is still uncertain. The data of Feldman & Boyer [28] on ADP (Mg^{2+})-induced inhibition of chloroplast ATPase and those of Drobinskaya *et al.* [29] on mitochondrial F_1 have been interpreted as the evidence that ADP inhibits ATPase when bound at a catalytic site in the absence of concomitant P_i binding. However, our data [14,32] and reports from other laboratories [30,31] suggest that ADP bound in the presence of Mg^{2+} at regulatory non-catalytic site(s) of mitochondrial F_1 is responsible for formation of the inhibited

Abbreviation used: CICCIP, carbonyl cyanide *m*-chlorophenylhydrazine.

* To whom correspondence should be addressed.

and slowly activated ATPase. Moreover, we have proposed that occupation of two nucleotide-binding regulatory sites on mitochondrial $F_0 \cdot F_1$ by either ADP or ATP in the presence of Mg^{2+} makes the third catalytic site to be in either ATP hydrolase or ATP synthase states respectively [32]. Much more experimental work seems to be necessary to prove or disprove either hypothesis.

Although the requirement of Mg^{2+} for the ADP-induced inhibition of F_1 -ATPases has long been recognized [10,12,13,33], most of the current studies on this phenomenon were focused on the interplay of the enzyme nucleotide-binding sites during the slow deactivation/activation process. Much less attention has been paid to the role of Mg^{2+} in the formation of the ADP-inhibited ATPase. Our previous studies on the ADP (Mg^{2+})-induced deactivation of $F_0 \cdot F_1$ ATPase of submitochondrial particles led us to conclude that the ADP- Mg^{2+} complex seems to be the unlikely species that binds to F_1 [13]. In the present paper we describe a procedure for the preparation of submitochondrial particles that are sensitive to Mg^{2+} -induced de-activation of their oligomycin-sensitive ATPase without added ADP. Using this preparation, several parameters of the Mg^{2+} - $F_0 \cdot F_1$ -ATPase complex relevant to the ADP(ATP)-dependent slow active/inactive enzyme transition were characterized.

MATERIALS AND METHODS

'AS' submitochondrial particles

Bovine heart submitochondrial particles freed of protein ATPase inhibitor (AS-particles) were prepared essentially as described by Racker & Horstman [34]. The particles were suspended in small volumes of 0.25 M-sucrose and stored in liquid N_2 . The ATPase activity of AS-particles measured with an ATP-regenerating system at 20 °C, pH 7.4, in the presence of 0.1 mM-ATP ($\sim K_m$) was 1.3–2.0 μ mol of ATP hydrolysed/min per mg of protein. The initial rate of ATP hydrolysis was insensitive to azide (0.1 mM) and was stimulated by sulphite (15–20% at 5 mM-sulphite) [14]. The preparations showed a lag phase in their ATPase activity after 2 min preincubation with low concentrations of ADP (1–2 μ M) in the presence of 2 mM- Mg^{2+} [11].

'ASD' submitochondrial particles

AS-particles (10 mg/ml) were incubated for 5 min at 20 °C in 1 ml of a mixture containing (final concentrations) 0.25 M-sucrose, 10 mM-Hepes/KOH buffer, pH 7.4, 20 μ M-EDTA, 1 mM- $MgCl_2$ and 5 μ M-ADP. Potassium EDTA solution was then added to the suspension to a final concentration of 3 mM and the suspension was applied to a Sephadex G-50 (coarse grade) column (1.5 cm $\times$ 30 cm) equilibrated with 75 mM-sucrose, 0.1 mM-KCl, 10 mM-Hepes/KOH buffer, pH 7.4, and 3 mM-potassium EDTA, and allowed to pass slowly through the column at room temperature ($\sim$ 1 h). The particles were collected ($\sim$ 3 ml), diluted 4-fold with a mixture used for column filtration and sedimented at 200 000 *g* for 45 min at 17 °C. The particles were suspended in 0.25 M-sucrose containing 20 μ M-EDTA and sedimented as described above. The final pellets (ASD-particles) were suspended in 0.25 M-sucrose containing 20 μ M-EDTA (8–10 mg/ml) and stored in liquid N_2 .

The ATPase activity of ASD-particles thus prepared, measured as described for AS-particles, was 1.3–1.9 μ mol of ATP hydrolysed/min per mg of protein. The initial rate of ATP hydrolysis was insensitive to azide (100 μ M) and was stimulated by sulphite (25–30% at 5 mM-sulphite). The preparation showed a lag phase in its ATPase activity after preincubation for 2 min with 1 mM- $MgCl_2$.

ATPase assay

The ATPase activity was measured at 20 °C as a decrease in the absorption of NADH at 340 nm in a reaction mixture containing (final concentrations) 0.25 M-sucrose, 0.1 M-KCl, 10 mM-Hepes/KOH buffer, pH 7.4, 0.1 mM-ATP, 2 mM- $MgCl_2$, 50 μ M-EDTA, 4 μ M-rotenone, 10 μ M-carbonyl cyanide *m*-chlorophenylhydrazone (CICCP), 1 mM-phosphoenolpyruvate, 0.16 mM-NADH, pyruvate kinase (5.5 units/ml) and lactate dehydrogenase (5 units/ml). The particles were rapidly thawed before use, diluted to a protein concentration of 1 mg/ml and stored at 20 °C during the experiments. The reaction was started by the addition of a suitable amount of the particles to the assay cuvette, and the decrease in absorption was monitored continuously for 2–3 min. The initial rates of the ATPase reaction were determined. No differences in the initial rates were observed when the concentration of azide in the assay mixture was doubled. In all experiments the ATPase activity was completely sensitive to oligomycin.

Other methods

The concentration of Mg^{2+} was determined by titration with EDTA at pH 10 with Eriochrome Black T as an indicator. The protein content was determined by the biuret method [35]. ATP (sodium salt), pyruvate kinase and lactate dehydrogenase were obtained from Reanal (Budapest, Hungary). The enzymes were extensively dialysed before use. ADP, NaN_3 , Hepes and EDTA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), $MgCl_2$ was from Merck (Darmstadt, Germany); phosphoenolpyruvate was from Reanal (Budapest, Hungary); CICCP was a Sigma product; rotenone was from Fluka (Buchs, Switzerland).

Other chemicals were of the purest grade commercially available.

RESULTS

De-activation of ATPase in different preparations of submitochondrial particles

We showed previously that two types of the soluble F_1 differ in their sensitivity towards the ADP (Mg^{2+})-induced de-activation can be prepared [12]. The F_1 prepared by the conventional procedure and precipitated from the ATP-containing solution was sensitive to the de-activation by Mg^{2+} alone, and de-activated ATPase was completely restored after treatment with phosphoenolpyruvate and pyruvate kinase. This result strongly suggests that bound ADP is involved in the Mg^{2+} -induced de-activation. Indeed, another type of F_1 , freed of bound nucleotides has been prepared, and the latter preparation showed no de-activation phenomena when preincubated with Mg^{2+} [12]. It appears that AS-particles prepared by the conventional procedure contain membrane-bound $F_0 \cdot F_1$ ATPase which is partly sensitive to ADP-plus- Mg^{2+} de-activation. When assayed in the presence of an ATP-regenerating system, ADP(Mg^{2+}) de-activation of ATPase appears as an ATP-dependent lag phase in its catalytic activity [13]. Since the rate of activation in the assay system is rather high (pseudo-first-order rate constant at 25 °C and at 100 μ M ATP is $\sim$ 1 min $^{-1}$), the accuracy of quantitative determination of the extent of de-activation is not too high. However, the de-activated 'state' of ATPase can be 'frozen' if azide is present in the assay system [14]. Thus the residual activity measured as the initial rate of the ATPase reaction in the presence of azide corresponds to the fraction of the active enzyme. This experimental approach was used in the present studies for quantification of the ADP(Mg^{2+})-induced de-activation reaction (Table 1). In order to obtain a homogeneous population of the membrane-bound $F_0 \cdot F_1$

Table 1. Sensitivity of F₀-F₁-ATPase of submitochondrial particles toward ADP(Mg²⁺)-induced de-activation

For preparation of AS- and ASD-particles, see the Materials and methods section. ASD(Mg²⁺)-particles were prepared exactly as described for ASD-particles, except that the treatment with EDTA was omitted and 2 mM-MgCl₂ was added to the column and washing buffer. The particles (1 mg/ml) were preincubated in a mixture containing 0.25 M-sucrose, 10 mM-Hepes/KOH buffer, pH 7.4, and 50 μM-EDTA (other additions are indicated) for 5 min at 20 °C and the initial rates of the ATPase reaction at 100 μM-ATP in the presence of 100 μM-NaN₃ were measured.

Preparation	Additions to the preincubation medium ...	ATPase activity (μmol/min per mg)		
		EDTA (3 mM)	MgCl ₂ (1 mM)	MgCl ₂ (1 mM) and ADP (1 μM)
1. AS-particles	†	1.64 (100 %)	0.85 (52 %)	0.08 (5 %)
2. ASD-particles		1.75 (100 %)	0.25 (14 %)	0.09 (5 %)
3. ASD(Mg ²⁺)-particles		1.60 (100 %)*	1.10 (69 %)	0.10 (6 %)

* Preincubated for 30 min before assay.

Fig. 1. Time course of Mg²⁺-induced de-activation of ASD-particles

(a) ASD-particles (0.8 mg/ml) were incubated at 20 °C in a mixture containing 0.25 M-sucrose, 10 mM-Hepes/KOH, pH 7.4, and EDTA (3 mM) or MgCl₂ (10, 20 and 100 μM; curves 1, 2 and 3 respectively). Suitable amounts of the mixture were withdrawn and the initial rates of ATP (100 μM) hydrolysis in the presence of 100 μM-NaN₃ [14] were measured; '100 %' corresponds to an ATPase activity of 1.9 μmol/min per mg. Slow de-activation was observed during preincubation of ASD-particles without EDTA and added Mg²⁺ which was prevented by EDTA. Direct determinations gave a value of 5 μM for contaminant Mg²⁺ originating mainly from Hepes. Because of this, EDTA was always added to the control samples, and, under these conditions, the ATPase activity of the particles was perfectly stable during 4 h incubation at 20 °C. The concentrations of Mg²⁺ in the incubation medium refer to their total (added and contaminant) content. (b) Semi-logarithmic plot of ATPase de-activation in the presence of 10 μM- (1), 20 μM- (2) or 100 μM- (3) MgCl₂. $V_{t-\infty}$ was determined as the residual azide-insensitive initial rate of ATP hydrolysis observed after preincubation of particles (as described) in the presence of 1 mM-Mg²⁺ for 30 min. The residual activity at infinite time ($v_{t-\infty}$) was subtracted from v_t values to determine the first-order rate constant. This residual activity (10–20 %) was apparently due to partial removal of ADP from the particles during Sephadex-gel filtration. Indeed, when 1 μM-ADP was added to the samples used for $v_{t-\infty}$ determination, no residual azide-insensitive ATP hydrolysis was observed in the assay system. Note that semi-logarithmic straight lines do not pass through the origin because 2 mM-Mg²⁺ was present in the assay mixture and relatively rapid additional de-activation took place before actual registration of ATPase (5 s after sample addition) was started.

sensitized to de-activation by Mg²⁺ (ASD-particles), the following procedure was worked out. AS-particles were first completely de-activated by preincubation with low ADP (1–2 μM) in the presence of Mg²⁺ and then treated with an excess of EDTA to remove Mg²⁺ and activate ATPase (see the Materials and methods section). As documented in Table 1, the particles thus prepared do not need added ADP to show almost complete de-activation by Mg²⁺. Similar to the soluble F₁ de-activated by Mg²⁺ alone [12] and to the AS-particles de-activated by ADP plus Mg²⁺ [13], ASD-particles were completely activated by treatment with phosphoenolpyruvate and pyruvate kinase, indicating that the

particles thus prepared contain tightly bound ADP, which is a prerequisite for Mg²⁺-induced de-activation. ASD-particles prepared as described were further used throughout the present studies.

Kinetics and equilibrium of Mg²⁺-induced ATPase de-activation of ASD-particles

As is documented in Table 1, ASD-particles preincubated with Mg²⁺ lose their ATPase activity. On the other hand the particles exhibit a normal initial ATPase activity despite the presence of 2 mM-Mg²⁺ in the assay mixture. The absence of instantaneous

Fig. 2. Dependence of the first-order de-activation rate constant on Mg^{2+} concentration

The hyperbolic dependence of pseudo-first-order rate constant (k_{in}^{app}) on Mg^{2+} concentration was determined and the data were plotted in the double-reciprocal form. According to Scheme 1 the intercepts of the straight line give the values $1/k_{in}$ (ordinate) and $-1/K_s^{Mg^{2+}}$ (abscissa). The values of $K_s^{Mg^{2+}}$ and k_{in} , equal to 10^{-4} M and 0.65 min^{-1} respectively were determined. For the experimental conditions, see Fig. 1.

Fig. 3. Equilibrium titration of ATPase de-activation by Mg^{2+}

ASD-particles (0.6 mg/ml) were preincubated with Mg^{2+} -EDTA (1 mM) buffer for 3 (●) or 4 (○) h as described in Fig. 1. The concentrations of free Mg^{2+} were calculated by the conventional procedure, using a K_d value for the Mg -EDTA complex of 1×10^{-6} M at pH 7.4; '100%' corresponds to an ATPase activity of $1.3 \mu\text{mol/min per mg}$, measured for the control samples incubated in the presence of 1 mM-EDTA.

inhibition of ATPase in the assay mixture could not be explained by instant displacement of tightly bound inhibiting ADP, since the maximal rate constant for the reactivation of $ADP(Mg^{2+})$ -inhibited ATPase was found to be a 2 min^{-1} [13]; this value is still much lower than the ATPase turnover. The most credible explanation for the apparent discrepancies between the strong inhibition of ATPase after preincubation with Mg^{2+} and the lack of inhibition in the assay mixture is that Mg^{2+} -induced de-activation itself is slow. The data presented in Fig. 1(a) show that

Fig. 4. EDTA-induced re-activation of Mg^{2+} -de-activated ATPase

ASD-particles (0.6 mg/ml) were de-activated at 20°C and at pH 7.5 in the presence of Mg^{2+} ($20 \mu\text{M}$) for 40 min. Potassium EDTA (○) or EDTA and HCl (●) were then added (indicated by arrow) to obtain 5 mM-EDTA (final concn.) and the indicated pH, and incubation was continued; 5 mM-EDTA was added before Mg^{2+} to the control samples incubated at pH 7.5 (○) and 6.5 (●); '100%' corresponds to an ATPase activity of $1.6 \mu\text{mol/min per mg}$.

de-activation of ASD-particle ATPase is, indeed, time- and Mg^{2+} concentration-dependent. The minimal scheme which accounts for these findings can be formulated as follows:

Scheme 1.

where D stands for ADP bound at one of the tight sites, and the complex marked with an asterisk is the de-activated ATPase. If a single site for Mg^{2+} is present, it might be predicted that an apparent first-order rate constant for the de-activation process determined from the semi-logarithmic plot shown in Fig. 1(b) would depend on Mg^{2+} concentration as a simple hyperbola with an apparent ' K_m ' equal to $K_s^{Mg^{2+}}$. Such a dependence with the parameters $K_s^{Mg^{2+}} = 1.1 \cdot 10^{-4}$ M and $K_{in.} = 0.65 \text{ min}^{-1}$ at pH 7.4, is shown in Fig. 2. According to Scheme 1 at equilibrium (i.e. at infinite time) the ratio:

$$\frac{[\text{ATPase}_{active}][Mg^{2+}]}{[\text{ATPase}_{inactive}]}$$

is determined by:

$$K_{eq.} = K_s^{Mg^{2+}} \times (k_{act.}/k_{in.})$$

Since the ratio $k_{act.}/k_{in.} \ll 1$ (an apparent 'irreversible' de-activation of ATPase by Mg^{2+} is evident at $[Mg^{2+}] \gg K_s^{Mg^{2+}}$; see Fig. 1a), the value of $K_{eq.}$ can be determined from the titration curve: ATPase activity versus Mg^{2+} concentration in the preincubation medium. The results of such a titration, presented in Fig. 3, gave a value for $K_{eq.}$ equal to 2×10^{-6} M. This gives the calculated $k_{act.}$ (taking $k_{on} = 0.65 \text{ min}^{-1}$ as determined in Fig. 2) equal to $1.1 \times 10^{-2} \text{ min}^{-1}$. To check the validity of the Scheme 1 and the correctness of our reasoning, the $k_{act.}$ value was directly measured. According to Scheme 1 de-activated ATPase ($*E:D_{Mg}$) should be re-activated with the first-order rate constant $k_{act.}$ if free Mg^{2+} is trapped by a chelating agent. The time course of EDTA-induced re-activation of the Mg^{2+} -de-activated ATPase

Fig. 5. Mg²⁺-induced de-activation of ATPase at different pH values

ASD-particles (0.6 mg/ml) were incubated in a mixture containing 0.25 M-sucrose, 5 mM-Mes, 5 mM-Hepes (pH as indicated) and 20 μ M-MgCl₂; 3 mM-EDTA was added to the control samples. '100 %' corresponds to an ATPase activity of 1.5 μ mol/min per mg.

Fig. 6. Re-activation of Mg²⁺-de-activated ATPase in the presence of P_i, ATP and sulphite

ASD-particles (0.6 mg/ml) were de-activated in the presence of 20 μ M-MgCl₂ as described in Fig. 1; 5 mM-EDTA and P_i (5 mM), ATP (1 μ M) or sulphite (5 mM) were then added where indicated and incubation was continued; 3 mM-EDTA was added to the control samples; '100 %' corresponds to an ATPase activity of 1.6 μ mol/min per mg.

at different pH values is shown in Fig. 4. The re-activation pattern corresponds to the first-order kinetics, with $k_{act.}$ equal to $7 \times 10^{-3} \text{ min}^{-1}$, i.e. a value which is very close to $1.1 \times 10^{-2} \text{ min}^{-1}$. The re-activation rate does not depend on the pH between 6.5 and 7.5. On the other hand, the rate and degree of Mg²⁺-induced de-activation are pH-dependent at any fixed concentration of Mg²⁺ (Fig. 5). These results suggest that the primary rapid binding of Mg²⁺ at the enzyme-specific site ($K_m^{Mg^{2+}}$; Scheme 1) is pH-dependent, whereas no protonation/deprotonation step is involved in the second isomerization step which produces inactive ATPase.

Effect of other specific ligands on Mg²⁺-F₀·F₁ interaction

An extremely slow re-activation of Mg²⁺-de-activated ATPase of ASD-particles in the presence of EDTA (Fig. 4) provides a simple model system for qualitative and quantitative analysis of

Fig. 7. Re-activation of Mg²⁺-de-activated ATPase by P_i

ASD-particles (0.5 mg/ml) were de-activated in the presence of 20 μ M-MgCl₂ for 1.5 h as described in Fig. 1; 5 mM-EDTA and different concentrations of P_i were added and the incubation was continued. The first-order constant for the re-activation rate were determined for each P_i concentration. The 'spontaneous' re-activation rate in the presence of EDTA only was subtracted from those measured in the presence of P_i. The ATPase activity reached after 1 h incubation in the presence of 5 mM-EDTA and 2 mM-P_i (the value $v_{t \rightarrow \infty}$ used to construct semi-logarithmic plots for the first-order-rate-constant determination) was 2.0 μ mol/min per mg. The inset shows a double-reciprocal plot for P_i-promoted re-activation.

the effects of some specific ligands on Mg²⁺-F₀·F₁ interaction. Fig. 6 demonstrates that addition of P_i, ATP or sulphite to the Mg²⁺-de-activated particles promotes re-activation induced by EDTA. One important conclusion from the data shown in Fig. 6 is that both P_i and ATP interact with the enzyme as free species (not as complexes with Mg²⁺). To assess further quantitative characteristics of such an interaction, the concentration-dependencies of the promotive effects of P_i and ATP were studied. The data presented in Fig. 7 demonstrate that the promoting effect of P_i is consistent with a single P_i-binding site on the enzyme (half-saturation at 1 mM-P_i) strongly interacting with Mg²⁺ bound at the inhibitory site. The same set of experiments were performed with ATP as a promoting ligand, and the combined data on the affinities and maximal re-activation rates reached at saturating P_i and ATP are given at Table 2. It seemed of interest to compare the maximal promoted re-activation rates reached in the experiments described in Fig. 6 with that obtained using pyruvate kinase (and phosphoenolpyruvate) as the activator [13] and ATP-dependent reactivation of ATPase in the assay system [13,32]. As we have pointed out previously [20], the only conceivable event during the pyruvate kinase/phosphoenolpyruvate-induced activation of ATPase in the presence of Mg²⁺ is the release of ADP from the specific inhibitory site. Thus it might be expected that the rate of pyruvate kinase/phosphoenolpyruvate-induced re-activation would be less than, or equal to, that observed in the presence of EDTA. Surprisingly, as depicted in Table 2, this is not the case, and pyruvate kinase re-activates ADP (Mg²⁺)-de-activated enzyme about 10 times faster than does EDTA. The likely explanation of these puzzling results seemed to be that pyruvate kinase-induced re-activation was always observed in the presence of Mg²⁺, because of the absolute requirement for Mg²⁺ for pyruvate kinase activity [36]. Thus it seems possible that free Mg²⁺ is a factor which promotes the release of tightly bound ADP from the specific site and subsequent rapid dissociation of Mg²⁺ from the inhibitor site. Although the direct experiments to

Table 2. Kinetic parameters of re-activation of Mg^{2+} -de-activated ATPase (20 °C, pH 7.4)

ASD-particles were de-activated with Mg^{2+} (20 μM) and re-activated as described in Figs. 6 and 7 by the addition of EDTA and promoting ligands. No EDTA was present when re-activation was initiated by pyruvate kinase (30 units/ml) and phosphoenolpyruvate (1 mM) in the presence of 2 mM- MgCl_2 (line 4). AS-particles de-activated by preincubation with ADP and 2 mM- MgCl_2 were used [32] when ATP-dependent re-activation in the ATPase assay system in the presence of 1 mM MgCl_2 was monitored (line 5).

Re-activating ligand	K_s (M)	$k_{\text{act.}}$ (min^{-1})*
1. EDTA (spontaneous reactivation)	—	0.007 (measured)
	—	0.011 (calculated)
2. P_i	1×10^{-3}	0.025
3. ATP	5×10^{-7}	0.06
4. Pyruvate kinase plus phosphoenolpyruvate	—	0.06
5. ATP (in the presence of Mg^{2+})†	$1 \times 10^{-4}\ddagger$	0.4 (de-activated with 1 μM -ADP)§
		1.0 (de-activated with 20 μM -ADP)§

* The value at saturating ligand concentration.

† ATP-dependent re-activation in the assay system.

‡ This value, measured at pH 8.0, is taken from [13].

§ Taken from [32].

check this proposal are somewhat difficult to perform, the results given in Table 1 (line 3) are in line with this proposal. When submitochondrial particles preincubated with 5 μM -ADP and 2 mM- Mg^{2+} were passed through the column equilibrated with 2 mM- Mg^{2+} the collected preparations showed a high sensitivity of their ATPase activity to preincubation with ADP.

DISCUSSION

It has been known for a long time that a number of the specific ligands, including the substrates (products) of oxidative phosphorylation, have a prominent effect on the $\text{ADP}(\text{Mg}^{2+})$ -induced de-activation of mitochondrial ATPase. De-activation was shown to be prevented and reversed by sulphite [14]. P_i also re-activates mitochondrial ATPase de-activated by ADP in the presence of Mg^{2+} [37]. The very complex behaviour of de-activation/re-activation processes, as related to ATP and ADP, has been observed. Re-activation of the $\text{ADP}(\text{Mg}^{2+})$ -de-activated ATPase is ATP-dependent [13], thus suggesting that binding or hydrolysis of ATP at a site other than that at which the inhibitory ADP binds is needed to re-activate ATPase. Moreover, the rate of ATP-dependent re-activation of the $\text{ADP}(\text{Mg}^{2+})$ -de-activated ATPase increases, when the concentration of ADP added to deactivate the enzyme, increases from 1 to 10 μM [32]. The latter finding has been interpreted as the functional interrelationship between three nucleotide-binding sites on F_1 [32].

It should be pointed out that, since most of the previous studies on de-activation/activation were focused on the ADP-specific inhibitory site and were performed in the presence of Mg^{2+} , the question as whether de-activation is caused by ADP or Mg^{2+} , or both, and whether re-activation is due to release of ADP or Mg^{2+} , or both, remained to be answered. The results of experiments with Mg^{2+} -induced de-activation of ASD-particles (Table 1) show that the ADP tightly bound to the $\text{F}_0\text{-F}_1$ -ATPase does not itself inhibit ATP hydrolysis. This is consistent with the earlier findings on soluble mitochondrial [11,12] or chloroplast [28,38] F_1 and chloroplast-bound ATP synthase [39].

The kinetics of Mg^{2+} -induced de-activation suggest the presence of a single inhibitory site for Mg^{2+} on ADP-loaded $\text{F}_0\text{-F}_1$. An important question related to the molecular organization of F_1 is: where is this site located? An obvious possibility would be that Mg^{2+} binds at the nucleotide-binding site where a potentially inhibitory ADP is located, thus producing a de-activated $^*\text{E}\cdot\text{ADP}\cdot\text{Mg}^{2+}$ complex. This proposal is consistent with the

suggestion that the total number of Mg^{2+} -binding sites on F_1 is equal to that of exchangeable nucleotides [25]. If the total number of Mg^{2+} -binding sites is more than that for nucleotides, as can be evidenced from the data obtained with rat liver F_1 [24,26] and chloroplast F_1 [40], there is another possibility which we would like to discuss here. It has been documented that azide inhibits mitochondrial ATPase via stabilization of the $\text{ADP}(\text{Mg}^{2+})$ -de-activated enzyme [14]. According to Kagawa *et al.* [41], a combination of pure $\alpha\beta$ -subunits of TF_1 from thermophilic ATP synthase symmetrically arranged in the hexamer is able to hydrolyse ATP, and this hydrolysis is azide-insensitive, in contrast with the azide-sensitive reaction catalysed by intact TF_1 [42]. Taken together, these results suggest two possibilities: either the γ -subunit induces asymmetry in the $3\alpha\cdot 3\beta\cdot 1\gamma$ structure, thus forming a Mg^{2+} -binding site on β -subunit, where the nucleotide-binding site is located [43], or a Mg^{2+} -specific inhibitory site is located on the γ -subunit, which is believed to be a link between F_1 and F_0 [44,45]. Hence a mechanism of ATP synthesis/hydrolysis coupled with vectorial proton movement through F_0 may be proposed, which involves $\text{Mg}^{2+}/\text{H}^+$ exchange at γ -subunit coupled with drastic changes in affinities for nucleotides and/or P_i at the specific catalytic (or non-catalytic regulatory) site(s). This proposal is in line with the earlier hypothesis on the participation of Mg^{2+} in the mechanism of oxidative phosphorylation [46,47].

The rapid release of bound ADP and Mg^{2+} after illumination of chloroplasts accompanied by the activation of ATPase has been demonstrated [39], and the authors concluded that the de-activated form of F_1 occurs only under laboratory conditions and may not have a role *in vivo*. It should be emphasized that, however similar the $\text{F}_0\text{-F}_1$ -ATP synthase complexes from a variety of sources appear to be, a number of properties for the chloroplast enzyme (activation by reducing agents [48], specificity of ATP hydrolysis to Ca^{2+} and Mg^{2+} [3]) and for mitochondrial F_1 (sensitivity to the protein inhibitor [49]) are quite different. Thus we believe that it could be premature to consider the $\text{ADP}(\text{Mg}^{2+})$ -induced de-activation of mitochondrial $\text{F}_0\text{-F}_1$ -ATPase just as a laboratory artefact. Moreover, it would not be judicious to relate directly the data obtained on the particle-bound oligomycin-sensitive ATP synthase (the present paper) to those obtained with soluble F_1 from bovine heart [22,43,50–53] or rat liver [24,26] just because the latter preparations, being active as ATPases, are not able to operate as ATP synthases, and, as we have discussed elsewhere [9,54], the entire mechanism

of ATP hydrolysis and synthesis catalysed by mitochondrial H⁺-ATPase may not be identical.

The data on the strong effects of ATP and P_i on the affinity of Mg²⁺ for the specific inhibitory site provide some new insights into the binding of substrates and products of oxidative phosphorylation with ATP synthase. The dissociation constant for single P_i-binding site in the absence of Mg²⁺ (1 mM at pH 7.4) is within the range of K_m for P_i for oxidative phosphorylation [55] and close to that deduced from the quantification of the protective effect of P_i on the modification of soluble F₁ by 7-chloro-4-nitrobenz-2-oxa-1,3-diazole [56]. This value is significantly higher than the values obtained in direct binding measurements [27]. The possibility that P_i binds to Mg²⁺-deactivated F₀·F₁-ATPase at the substrate-binding site of ATP synthase seems to be very likely. It has been argued that de-activated ATPase arises as a result of tight binding of ADP at the catalytic site without concomitant P_i when the enzyme is pre-incubated with ADP and Mg²⁺, or during the steady-state ATP hydrolysis, when P_i dissociates before ADP, leaving the enzyme with ADP at the catalytic site in the form which is susceptible to Mg²⁺-induced de-activation [28,29]. This proposal seems to be supported by original observations of the activating effect of P_i on ADP(Mg²⁺)-de-activated ATPase [10,37], which was confirmed and extended in the present studies. However, it has been shown that de-activated ATPase still arises during the steady-state hydrolysis of ATP in the presence of 10 mM-P_i, and no changes in K_m for ATP or K_i for ADP in the presence of P_i were found [37]. It is remarkable that P_i has no inhibitory effect on mitochondrial ATPase [37]. Should the mechanism of ATP hydrolysis and synthesis catalysed by F₀·F₁-ATP synthase be identical and include a step where P_i reversibly associates/dissociates with the enzyme, one must expect the inhibition of ATP hydrolysis by P_i to take place. Taking into consideration the slowness of ATPase activation under any experimental conditions studied so far (see Table 2), it becomes certain that the *E:^D_{Mg} complex cannot be an intermediate of the ATPase reaction. It has been shown that ADP(Mg²⁺-de-activated particles catalyse ATP synthesis without any lag phase [57], as might be expected if one assumes that de-activated ATPase is an active ATP synthase.

ATP was shown to bind to the de-activated enzyme without free Mg²⁺, and this binding accelerates the release of Mg²⁺ from an inhibitory site. It is generally accepted that the ATP-Mg²⁺ complex is the substrate for F₁-type ATPases [58–61]. It appears, then, that free ATP, which promotes the EDTA-induced re-activation, binds to ATPase at a non-catalytic (regulatory) site. It is not clear whether ADP, which is a prerequisite for the Mg²⁺-induced de-activation, is bound as an Mg²⁺-complex or in the free form. If a symmetry between the two regulatory sites exists, as has been briefly discussed in our previous work [32], one may propose the existence of two free nucleotide-binding sites, one catalytic Mg²⁺-nucleotide-binding site and one Mg²⁺-specific ATPase inhibitory site, on F₀·F₁-ATP synthase.

We are indebted to Mrs. R. Birnova for linguistic advice.

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Received 31 May 1990/10 October 1990; accepted 14 November 1990