Pre-Steady-State Studies of the Adenosine Triphosphatase Activity of Coupled Submitochondrial Particles. Regulation by ADP†

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Received August 27, 1987; Revised Manuscript Received April 19, 1988

ABSTRACT: ATPase activities were measured in 10 mM $MgCl_2$, 5 mM ATP, 1 mM ADP, and 1 μM FCCP with submitochondrial particles from bovine heart that had been stimulated by $\Delta\tilde{\mu}_{H^+}$-forming substrates and with particles whose natural inhibitor protein was partially removed by heating. The activities were not linear with time. With both particles, the rate of ATP hydrolysis in the first 5 s of incubation was 6- to 7-fold greater than that in the steady state. Pre-steady-state and steady-state kinetic studies showed that the decrease of ATPase activity was due to the binding of ADP in a high-affinity site of the enzyme ($K_{0.5}$ of 10 μM). Inhibition of ATP hydrolysis was accompanied by the binding of approximately 1 mol of ADP/mol of particulate F_1; 10 μM ADP gave half-maximal binding. ADP could be replaced by IDP, but with an affinity 50-fold lower ($K_{0.5}$ of 0.5 mM). Maximal inhibition by ADP and IDP was achieved in less than 5 s. Inhibition was enhanced by uncouplers. Even in the presence of pyruvate kinase and phosphoenolpyruvate, the rates of hydrolysis were about 2.5-fold higher in the first seconds of reaction than in the steady state. This decrease of ATPase activity also correlated with the binding of nearly 1 mol of ADP/mol of F_1. This inhibitory ADP remained bound to the enzyme after several thousand turnovers. Apparently, it is possible to observe maximal rates of hydrolysis only in the first few catalytic cycles of the enzyme.

The membranes of mitochondria, chloroplasts, and bacteria contain a proton-translocating coupling factor or ATP synthase. This ATP synthase is composed of a hydrophobic portion, F_0, which is responsible for proton translocation, and a hydrophylic moiety, F_1,[1] which catalyzes the synthesis and hydrolysis of ATP (Hatefi, 1985). The F_1 portion is composed by five types of subunits arranged in a stoichiometry of α_3, β_3, γ, δ, and ϵ (Hatefi, 1985). In addition, the ATP synthase possesses a detachable low molecular weight protein that has been referred to as the natural ATPase inhibitor (Pullman & Monroy, 1963). This protein binds to the β subunit of F_1 (Klein et al., 1980) and inhibits both ATP synthesis and hydrolysis (Gômez-Puyou et al., 1979; Harris et al., 1979); its inhibitory action in particulate F_1-ATPase is released by membrane energization (Van de Stadt et al., 1973; Gômez-Puyou et al., 1979; Tuena de Gômez-Puyou et al., 1980; Dreyfus et al., 1981; Schwerzmann & Pedersen, 1981; Power et al., 1983; Tuena de Gômez-Puyou et al., 1984b; Husain, et al., 1985; Schwerzmann & Pedersen, 1986).

In chloroplasts another important point of control of $\Delta\tilde{\mu}_{H^+}$-dependent ATPase activity has been demonstrated. Under energized conditions, a high hydrolytic activity becomes apparent when nucleotide diphosphates are released from the enzyme (Harris & Slater, 1975; Shoshan & Selman, 1979; Strotmann et al., 1979); this release is related to the existence of electrochemical H^+ gradients, since the collapse of $\Delta\tilde{\mu}_{H^+}$ induces binding of nucleotide diphosphate and consequent inhibition of hydrolytic activity (Bar-Zvi & Shavit, 1982). Mitochondrial soluble F_1 has six adenine nucleotide binding sites (Slater et al., 1979; Kironde & Cross, 1986), and it has been demonstrated that ADP promotes a decrease in ATPase activity (Rosing et al., 1975; Harris, 1978; Fitin et al., 1979; Vasilyeva et al., 1980; di Pietro et al., 1980; Vasilyeva et al., 1982; Drobinskaya, et al., 1985). However, these studies have been made with the soluble enzyme (Rosing et al., 1975; di Pietro et al., 1982; Vasilyeva et al., 1982; Drobinskaya et al., 1985) or uncoupled particles (Fitin et al., 1979; Vasilyeva et al., 1980), and thus it was not possible to assess the contribution of $\Delta\tilde{\mu}_{H^+}$ over this regulatory mechanism. This work was concerned with the role of the electrochemical H^+ gradients and ADP on the regulation of ATPase activity of coupled submitochondrial particles. These studies were made at pre steady state of ATP hydrolysis by measuring ATP hydrolysis in times of seconds, and by using high concentrations of particles. It was found that even in the presence of an ADP trap, the binding of one ADP per particulate F_1 provokes a severalfold decrease of ATPase activity in less than 5 s and that this effect is potentiated by uncouplers. The results imply that ATPase activity at the steady state is always controlled by ADP bound to the enzyme.

Materials and Methods

Bovine heart mitochondria were prepared according to Low and Vallin (1963). Heavy mitochondria were stored at −70

† This investigation was supported by grants from the Organization of American States, Ricardo J. Zevada Foundation, and CONACyT, México.

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[1] Abbreviations: IP, natural inhibitor protein; F_1, bovine heart mitochondrial F_1-ATPase; CF_1, chloroplast F_1-ATPase; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; 1799, α,α'-bis(hexafluoroacetonyl)acetone; Ap5A, P^1,P^5-bis(5′-adenosyl)pentaphosphate; oxonol di-BaC_4-(3)-, bis[1,3-dibutylbarbituric acid (5)] trimethine oxonol; PEP, phosphoenolpyruvate; PK, pyruvate kinase; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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°C. Mg-ATP submitochondrial particles were prepared as described (Lee & Ernster, 1967). The hydrolytic activity of these particles (approximately 0.3 μmol of $P_i \cdot min^{-1} \cdot mg^{-1}$) is largely controlled by IP. Coupled submitochondrial particles largely devoid of the inhibitory action of IP on their F_1-ATPase with a hydrolytic activity of 5–6 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ were prepared by a 4–5-h incubation at 38 °C of a suspension of Mg-ATP particles at 50 mg of protein/mL in a medium containing 50 mM KH_2PO_4, 0.25 M sucrose, and 95% D_2O (pD 7.8) (Beltrán et al., 1986). These particles are referred to throughout the paper as *noncontrolled particles*. These particles are coupled, as evidenced by their capacity to synthesize ATP in presence of an ATP and to build up a membrane potential to an extent similar to that of Mg-ATP particles [not shown, but see Beltrán et al. (1986)].

Adenosine triphosphatase activity was assayed by three different methods. In the presence of added ADP, the activity was assayed by measuring the release of [^{32}P]P_i from [γ-^{32}P]ATP. [^{32}P]P_i was extracted from the assay medium as phosphomolybdate complex with butyl acetate, and an aliquot of organic phase was counted in a liquid scintillation counter (Tuena de Gómez-Puyou et al., 1984a). [γ-^{32}P]ATP was prepared according to Glynn and Chappell (1964). In the absence of added ADP, the ATPase activity was assayed by recording NADH oxidation at 340 nm (Pullman et al., 1960), or by assay of pyruvate formed during ATP hydrolysis. In the latter case, the incubation mixture contained the concentrations of phosphoenolpyruvate and pyruvate kinase indicated under Results. The amount of pyruvate kinase included is expressed in units (micromoles of pyruvate formed per minute) determined in the conditions of the experiment. In these conditions, the K_m for ADP was 310 μM and the V_{max} was 70 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. These were determined as described by Plowman and Krall (1965), and the results are comparable to those obtained by the authors. The reaction was stopped with 1 N perchloric acid, and after centrifugation, an aliquot of the supernatant was neutralized with KOH. Perchlorate was removed by centrifugation, and an aliquot of the supernatant was diluted in a medium containing 50 mM Tris–acetate buffer, pH 7.2, 10 mM $MgCl_2$, 30 mM KCl, and 0.2 mM NADH. The absorbance was read at 340 nm before and after adding 10 units of lactate dehydrogenase. From the difference of the two readings, the amount of ADP generated during ATP hydrolysis was calculated. In most of the experiments, activity was determined in reaction times of 5 and 10 s. The reaction was started and stopped by making the indicated additions to tubes that were under constant stirring. Duplicate or triplicate samples were always run, and the difference was always less than 10%. All the experiments described were the average of at least three determinations with different preparations. In all experiments carried out to determine [γ-^{32}P]ATP hydrolysis or pyruvate formation, blanks that contained all the components of the reaction mixture were made; in these trichloroacetic acid or perchloric acid was added before the particles.

Binding to ADP to F_1 of noncontrolled submitochondrial particles was carried out as described by Myers and Boyer (1983). The standard procedure for determination of [^{3}H]-ADN binding to F_1 of submitochondrial particles was as follows. Particles (0.5 mg of protein) were incubated in 100 μL of a medium that contained 20 mM Tris–acetate, pH 7.2, 200 mM sucrose, 10 mM $MgCl_2$, 20 mM KCl, 50 μM Ap5A, 50 μM carboxyatractyloside, and either [2,8-^{3}H]ATP or [2,3-^{3}H]ADP. When [^{3}H]ADP binding was measured, the mixture also contained 20 mM glucose, 50 units of hexokinase, and 50 μM [^{3}H]ADP unless otherwise indicated. When [^{3}H]ATP was used as a substrate for binding, the mixture contained 50 μM [^{3}H]ATP, 10 units of pyruvate kinase, and 20 mM phosphoenolpyruvate. The specific activity of [2,8-^{3}H]ADN was 4×10^5 cpm/nmol. After incubation for various times, the mixtures were filtered twice through centrifuge columns (Penefsky, 1977) equilibrated with 20 mM Tris–acetate, pH 7.2, 200 mM sucrose, and 10 mM $MgCl_2$. Aliquots of the filtrate were used to quantitate protein and radioactivity. To calculate the ratio of [^{3}H]ADN bound per ATPase, a value of 0.42 nmol of ATPase/mg of protein of submitochondrial particles was used (Harris et al., 1977). Protein was determined as described (Lowry et al., 1951), on the basis of bovine serum albumin as standard, by using an extinction coefficient of $E_{280nm} = 6.67\ mg^{-1} \cdot cm^{-1}$ (Foster & Sherman, 1956).

Identification of [^{3}H]ADN Bound to F_1 of Submitochondrial Particles. The composition of [^{3}H]ADN bound to submitochondrial particles was assayed by addition of perchloric acid (0.5 M final concentration) to the filtrate obtained from the centrifuge columns. After addition of perchloric acid, 50 μL of 300 μM ATP and ADP were added. The supernatant was neutralized with KOH and perchlorate removed. Nucleotides were separated by HPLC (Waters) in a Radial PAK SAX P 3082A01 column (Waters) that was eluted with 0.25 M KH_2PO_4 and 0.5 M KCl, pH 5.0. Fractions were collected, and radioactivity was determined. In all experiments 8–10% of the total radioactivity corresponded to [^{3}H]AMP.

Membrane Potential. This was monitored with oxonol di-BaC_4-(3)$^-$ as described by Beltrán et al. (1986), except that the incubation media employed corresponded to those used in this work.

Results

Stimulation of ATPase Activity of Mg-ATP Submitochondrial Particles by $\Delta\tilde{\mu}_{H^+}$. The incubation of Mg-ATP particles with oxidizable substrates, such as succinate or NADH (Van de Stadt et al., 1973), or with Mg-ATP (Power et al., 1983), promotes an increase in ATPase activity due to release of the inhibitory action of IP on F_1-ATPase as induced by energization. We observed that the hydrolytic activity of Mg-ATP particles increased by preincubation with ATP, but also with ITP (Table I). When this effect of NTP was studied, it was considered that, during the preincubation of particles for various times with NTP, different amounts of NDP are produced and that these may affect the hydrolytic activity of the particles when transferred to the assay medium. This would seem to be more important for the case of ADP (Penefsky, 1979). To overcome this difficulty, the hydrolytic activity was measured in two different conditions, i.e., spectrophotometrically in the presence of an ADP trapping system and by hydrolysis of [γ-^{32}P]ATP in the presence of a high concentration of added ADP.

The data shown in Table I illustrate the results obtained with the two methods. As expected in the two conditions, FCCP in the reaction mixture produced an increase in ATPase activity. However, it was surprising that higher ATPase activities were detected in the presence of added ADP than in media containing an ADP trap (Table I). As shown below, the difference was due to the difference in the incubation time in the two experimental conditions. Nevertheless, experiments were carried out to explore this behavior and to ascertain if the inhibitor protein had a bearing on this phenomenon.

To this purpose, controlled particles were preincubated with either succinate, ATP, or ITP for various times. Their activity increased to a maximum in about 10 min (Figure 1A). As

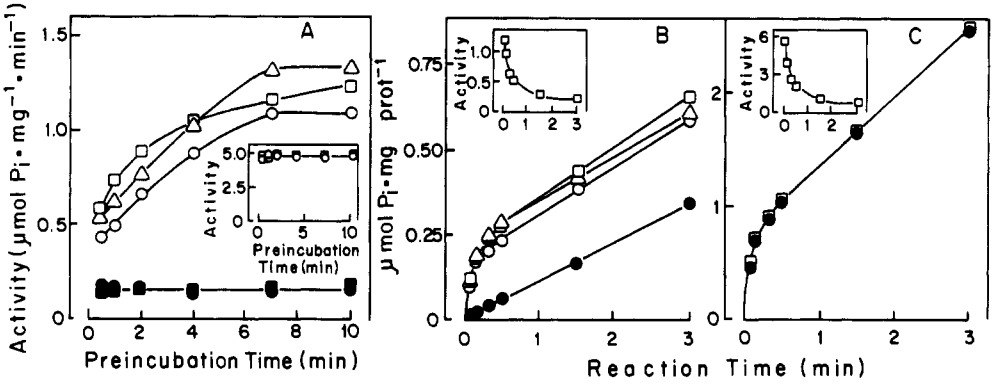

FIGURE 1: (A) Kinetics of stimulation of ATPase activity by $\Delta\tilde{\mu}_{H^+}$-forming substrates. Mg-ATP submitochondrial particles were preincubated at a concentration of 5 mg of protein/mL in a medium containing 50 mM Tris–acetate buffer (pH 7.2), 10 mM $MgCl_2$, and 0.2 M sucrose (●). The mixture was supplemented with 8 mM ATP (△), 8 mM ITP (○), 8 mM succinate (□), or 8 mM succinate plus 1 μM FCCP (■). At different preincubation intervals, aliquots of 0.06 mL were added to 0.24 mL of a medium to yield the final concentrations: 50 mM Tris–acetate buffer (pH 7.2), 10 mM $MgCl_2$, 30 mM KCl, 50 μM Ap5A, 5 mM P_i, 1 μM FCCP, 2 mM ADP, and 5 mM [γ-^{32}P]ATP. After 10 s, the reaction was stopped by addition of trichloroacetic acid. Both preincubation and reaction were performed at 30 °C. The experimental conditions of the inset were as described above, except that noncontrolled particles (see Materials and Methods) were used. (B) Decay of ATPase activity of stimulated Mg-ATP particles in the presence of ADP. The experimental conditions were the same as in panel A, except that the particles were preincubated in different media during 10 min and that ATP hydrolysis was stopped at the times indicated. The inset shows the specific activities (μmol·mg^{-1}·min^{-1}) at the times indicated. (C) Decay of ATPase activity of noncontrolled particles. The experimental conditions were the same as in panel B, except that noncontrolled particles preincubated in the absence (●) or presence of (□) of succinate were used.

Table I: ATPase Activity of Mg-ATP Particles Preincubated with ATP, ITP, or Succinate[a]

	ATPase act. (μmol of P_i·mg^{-1}·min^{-1})			
	with 2 mM ADP		with PEP + PK	
preincubation	−FCCP	+FCCP	−FCCP	+FCCP
	0.11	0.13	0.25	0.35
ATP	0.20	1.15	0.42	0.83
ITP	0.18	1.00	0.27	0.63
succinate	0.28	1.10	0.42	0.79

[a] Mg-ATP particles (5 mg of protein) were preincubated in a medium containing 50 mM Tris–acetate buffer (pH 7.2), 10 mM $MgCl_2$, 0.2 M sucrose, and, when indicated, 8 mM ATP, 8 mM ITP, or 8 mM succinate. After 5 min of preincubation under constant stirring, ATPase activity was measured in the presence of added ADP, or spectrophotometrically in the presence of pyruvate kinase and phosphoenolpyruvate (PEP). In the former, an aliquot of 30 μL was diluted 10-fold in a mixture that contained (final concentrations) 50 mM Tris–acetate buffer (pH 7.2), 10 mM $MgCl_2$, 30 mM KCl, 50 μM Ap5A, 0.2 M sucrose, 5 mM P_i, 0.5 mM malonate, 2 mM ADP, 5 mM [γ-^{32}P]ATP (0.25 Ci/mol), and, where indicated, 1 μM FCCP; the reaction was stopped after 10 s, and [^{32}P]P_i formed was determined. For the spectrophotometric recording of activity the same media were used except that nonradioactive ATP was used, and ADP was substituted for 1 mM PEP, 4 units of pyruvate kinase, 10 units of lactate dehydrogenase, 2 μg/mL rotenone, and 0.2 mM NADH. In this assay 30 μg of particle protein was added to 3 mL of reaction media. The temperature in the preincubation and reaction was 30 °C.

Table II: Decrease of ATPase Activity by ADP at Various Intervals of Reaction[a]

expt	time (s)	total ADP (mM)	ADP (mM)	sp act. (μmol of P_i·mg^{-1}·min^{-1})
1	0	1		
	5	1.56	0.56	6.7
	10	1.90	0.34	4.1
	20	2.43	0.53	3.2
	30	2.70	0.27	1.6
2	0	2		
	5	2.49	0.49	5.9
	10	2.77	0.28	3.4
	20	3.13	0.36	2.2
	30	3.30	0.17	1.0

[a] The ATPase activity of noncontrolled particles (1 mg/mL) was assayed at 30 °C in media that contained 50 mM Tris–acetate (pH 7.2), 30 mM KCl, 50 μM Ap5A, 10 mM $MgCl_2$, 0.2 M sucrose, 1 μM FCCP, 5 mM [γ-^{32}P]ATP, and 1 or 2 mM added ADP as indicated. At the times shown the reaction was stopped and the amount of [^{32}P]P_i formed was determined. From this value the total amount of ADP and that formed at various times were calculated.

expected, the ATPase activity of noncontrolled particles was not increased by any of the latter additions (inset). In a second experiment, the ATPase activity of particles whose ATPase had been stimulated in the latter conditions for 10 min was measured in the presence of added ADP in short incubation times (Figure 1B). The particles exhibited a high ATPase activity in the first 5 s of incubation that was followed by a slower rate of hydrolysis (Figure 1B). In view of the results that are described below, it should be noted that preincubation with Mg-ATP was carried out in the absence of uncouplers. In these conditions, the particles formed ADP and also a membrane potential that persisted for more than 10 min (not shown). The ATPase activity of noncontrolled particles, which are largely devoid of inhibitor protein and which had been preincubated with or without succinate, also declined with time. The insets of Figure 1 (B and C) show that the percentage decrease of specific activities with reaction time was markedly similar in both stimulated particles and particles that had been largely depleted of inhibitor protein. This indicated that the inhibitor protein was not involved in the progressive diminution of ATPase activity with reaction time.

In order to evaluate the role of ADP in the inhibition of ATPase activity, ATP hydrolysis of noncontrolled particles was measured at various times in mixtures that contained 1 and 2 mM added ADP. Table II shows that the specific activity of hydrolysis at all reaction times studied was lower in mixtures that had been supplemented with 2 mM ADP. However, the data also indicate that, in addition to product inhibition, ADP induced a progressive inhibition of the hydrolytic activity that seemed to depend on the turnover of the enzyme, or on time of exposure to ADP. Indeed, at approximately equal concentrations of total ADP (compare data of experiments 1 and 2), the activity was lower in the enzyme that had undergone a higher number of turnovers, or alternatively a longer time of incubation with medium ADP.

Effect of Preincubation of Noncontrolled Particles with ADP and/or FCCP on Their ATPase Activity. In order to explore the effect that ADP exerts on the hydrolytic activity

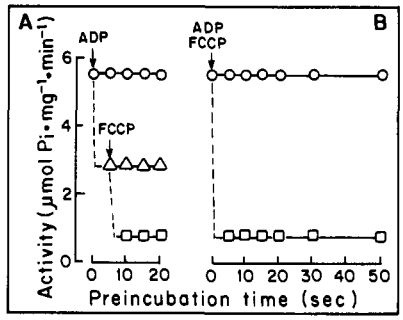

FIGURE 2: Effect of preincubation of ADP with and without FCCP on the ATPase activity of noncontrolled particles. Noncontrolled particles (0.3 mg of protein) (see Materials and Methods) were preincubated in 0.24 mL of a medium containing 62.5 mM Tris–acetate buffer (pH 7.2), 37.5 mM KCl, 62.5 μM Ap5A, 12.5 mM $MgCl_2$, and 0.2 M sucrose (○). In (A), when indicated by an arrow (↓), ADP (△) or FCCP (□) was added. In (B), ADP and FCCP (□) were added simultaneously. At the preincubation times indicated, the reaction was started by addition of a solution containing ADP, FCCP, and [γ-^{32}P]ATP (○), FCCP and [γ-^{32}P]ATP (△), or [γ-^{32}P]ATP (□). When ATP hydrolysis was started, the final concentrations of ADP, FCCP and [γ-^{32}P]ATP in the reaction were 1 mM, 1 μM, and 5 mM, respectively. The reaction was stopped at a fixed time of 10 s by addition of trichloroacetic acid. Both preincubation and reaction were performed at 30 °C.

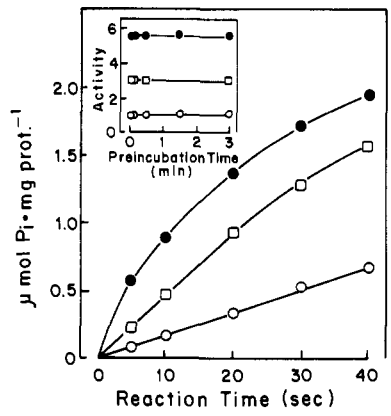

FIGURE 3: Decay of ATPase activity of noncontrolled particles incubated in different media. Noncontrolled particles (0.3 mg of protein) (see Materials and Methods) were preincubated in 0.25 mL of a medium containing 60 mM Tris–acetate buffer (pH 7.2), 36 mM KCl, 60 μM Ap5A, 12 mM $MgCl_2$, and 0.2 M sucrose with no further addition (●) or with either 1.2 mM ADP (□) or 1.2 mM ADP plus 1.2 μM FCCP (○). After 5 s of preincubation, ATP hydrolysis was started by addition of 0.05 mL of a solution containing ADP, FCCP, and [γ-^{32}P]ATP (●); FCCP and [γ-^{32}P]ATP (□); or [γ-^{32}P]ATP (○). When ATP hydrolysis was started, the final concentrations of ADP, FCCP, and [γ-^{32}P]ATP in the reaction were 1 mM, 1 μM, and 5 mM, respectively. At the times indicated, the reactions was stopped by addition of trichloroacetic acid. Both preincubation and reaction were performed at 30 °C. Inset: Effect of preincubation time on the ATPase activity. The experimental conditions were the same as in the main figure, except that particles were preincubated in different media for the times indicated and ATP hydrolysis was stopped at a fixed time of 10 s of reaction.

of the enzyme, coupled noncontrolled particles were preincubated in media that contained either ADP or FCCP plus ADP (Figure 2). After this time, aliquots of the suspensions were added to mixtures that contained [γ-^{32}P]ATP and FCCP, and their ATPase activity was determined in 10 s of reaction time. Particles preincubated in the absence of ADP exhibited an activity of approximately 6 μmol·min^{-1}·mg^{-1}. In contrast, the activity of particles preincubated with ADP for 5 s had an ATPase activity of about 3 μmol·min^{-1}·mg^{-1} (Figure 2). The subsequent addition of FCCP to these particles caused a further diminution of the activity to a value of less than 1 μmol·min^{-1}·mg^{-1} (Figure 2). Moreover, the preincubation of particles for 5 s with both ADP and FCCP caused a drop in the activity to a value of approximately 1 μmol·min^{-1}·mg^{-1} (Figure 2B). Thus it would seem that maximal inhibition by ADP required the abolition of a H^+ gradient. As during the preincubation no $\Delta\tilde{\mu}_{H^+}$-forming substrates were included, there was the possibility that, through myokinase activity, particles incubated with ADP could give rise to ATP. This ATP in turn could be hydrolyzed, yielding an electrochemical H^+ gradient. Indeed, through myokinase activity our preparations could form ATP with 1 mM ADP at a rate of 70 nmol·min^{-1}·mg^{-1}; this rate was inhibited by 50 μM Ap5A to a value of about 20 nmol·min^{-1}·mg^{-1}. However, it was found that in the standard experimental systems (Figure 2A), and as monitored with oxonol (Beltrán et al., 1986), no formation of membrane potential was detected with ADP. The experiments outlined in Figure 3 illustrate the changes in activity of particles preincubated for 5 s with ADP and ADP + FCCP in reaction times of 5–50 s. The low activity attained by preincubation of particles with ADP + FCCP remained constant throughout the time of the reaction. The preincubation of the particles with ADP alone promoted a decrease of the activity to about half of its original value when this activity was determined in times shorter than 30 s. The ATPase activities attained at times higher than 30 s were progressively lower (not shown). Five seconds of preincubation in either of the assayed conditions sufficed to attain equilibrium between the different forms of the enzyme, since preincubation times up to 3 min did not produce further changes in ATPase activity (inset of Figure 3). The findings of Figures 2 and 3 indicate that maximal inhibition of hydrolysis required preincubation with ADP and FCCP. This may explain why controlled particles that had been preincubated with Mg^{2+} + ATP (in which substantial amounts of ADP and a membrane potential were formed) presented a burst of high hydrolytic activity upon the addition of FCCP and the rest of the hydrolytic media (Figure 1B).

Binding of ADP and Inhibition of Hydrolysis. In order to estimate the ADP concentration required to inhibit ATPase activity and to explore if IDP also had a similar effect, noncontrolled particles were incubated in the presence of FCCP and different NDP concentrations for 5 s. Thereafter, ATPase activity was assayed in a reaction time of 10 s in the presence of 1 mM ADP or 32 mM IDP (Figure 4A). These high NDP concentrations in the assay medium were used to circumvent the problem of carrying different amounts of NDP from the preincubation to the reaction media, and they corresponded to the highest concentrations of NDP included in the preincubation media. The data indicate that half-maximal inhibition was obtained with 10 μM ADP and 0.5 mM IDP (Figure 4A). In order to explore if inhibition was due to the binding of the nucleotide, noncontrolled particles were incubated with various concentrations of [^{3}H]ADP in conditions similar to those in which the effect of ADP on hydrolysis was studied, and the amount of [^{3}H]ADP bound was determined. The results (Figure 4B) show that maximal inhibition of ATPase activity correlated with the binding of 1 mol of ADP/mol of ATPase. Moreover, with 10 μM ADP half-maximal inhibition of hydrolysis and half-maximal binding were attained.

ATPase Activity of Noncontrolled Particles in the Presence of a Medium ADP Trapping System. The aforementioned data indicate that the rate of ATP hydrolysis decays with time of incubation in media that contain added ADP and that inhibition of hydrolysis correlates with binding of ADP to F_1. It was thought that the role of ADP in the control of ATPase activity could be better characterized by assaying the variations

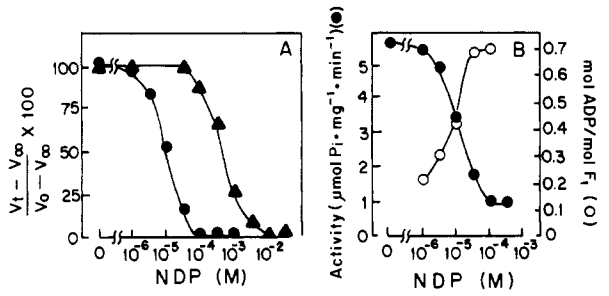

FIGURE 4: (A) Effect of preincubation of noncontrolled particles with different NDP concentrations. Noncontrolled particles (0.3 mg of protein) (see Materials and Methods) were preincubated in 0.25 mL of a medium containing 60 mM Tris–acetate buffer (pH 7.2), 36 mM KCl, 60 μM Ap5A, 12 mM $MgCl_2$, 0.2 M sucrose, 1 μM FCCP, and ADP (●) or IDP (▲) at the concentrations shown on the abscissa. After 5 s of preincubation, ATP hydrolysis was started by addition of 0.05 mL of solutions that contained [γ-^{32}P]ATP and ADP (●) or IDP (▲). After these additions, the final concentrations of the latter components in the reaction media were 5 mM [γ-^{32}P]ATP, 1 mM ADP (●), or 32 mM IDP (▲). The reaction was arrested after 10 s of reaction time. Both preincubation and reaction were performed at 30 °C. V_0 is the ATPase activity of particles preincubated in the absence of NDP, V_t is the activity of particles preincubated in different NDP concentrations, and V_∞ is the remaining activities. (B) Correlation between the inhibition of ATP hydrolysis and the binding of ADP. For ATPase activities the experimental conditions were the same as in panel A. For ADP binding 0.5 mg of noncontrolled particles were incubated in 0.1 mL of the medium described in (A) with the concentrations of [2,8-^{3}H]ADP shown in the abscissa. The binding was determined after 15 s as descibed under Materials and Methods.

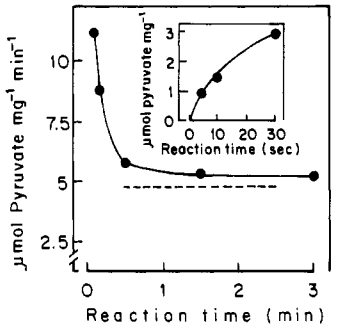

FIGURE 5: ATPase activity in the presence of ADP trapping system of noncontrolled particles preincubated in different media. Noncontrolled particles (0.3 mg of protein) (see Materials and Methods) were preincubated in 0.25 mL of a medium containing 60 mM Tris–acetate buffer (pH 7.2), 36 mM KCl, 60 μM Ap5A, 12 mM $MgCl_2$, and 0.2 M sucrose. After 5 s of preincubation, ATP hydrolysis was started by addition of 0.05 mL of solutions that contained FCCP, ATP, PEP, and PK. After the addition, the final concentrations of the latter components in the reaction media were 1 μM FCCP, 5 mM ATP, 20 mM PEP, and 80 units/mL pyruvate kinase. At the times indicated (●), the reaction was stopped by addition of perchloric acid, and pyruvate formed was determined as described under Materials and Methods. Both preincubation and reaction were performed at 30 °C. The ATPase activity of particles preincubated in the same conditions was also recorded spectrophotometrically, by adding an aliquot of the preincubation mixture (12 μL) to a 3-mL cuvette (see Materials and Methods). The specific activity obtained with this method is illustrated by the dashed line. The inset shows the amount of pyruvate generated at the times indicated.

that occur in the initial events of ATP hydrolysis in the presence of pyruvate kinase and PEP (Figure 5), and by measuring the amount of ADP that binds to F_1 in these conditions (Table III, Experiment 1). ATPase activity was measured in the presence of high concentrations of particles; in these conditions amounts of product formed in the initial seconds of reaction could be accurately measured. When the ATPase activity of noncontrolled particles was continuously monitored spectrophotometrically in the interval of 0.5–2.5 min, no change in ATPase activity took place (dashed line of

Table III: Binding and Release of ADP during Hydrolysis of ATP[a]

expt	time (min)	[^{3}H]ADN bound (mol/mol of F_1)
1	0.3	1.16
	2	1.17
	3	1.28
2	0	0.76
	1	0.57
	3	0.50

[a] In experiment 1, 0.5 mg of noncontrolled particles was incubated in 0.1 mL of 200 mM sucrose, 20 mM Tris–acetate (pH 7.4), 10 mM $MgCl_2$, 30 mM phosphoenolpyruvate, 3 units of pyruvate kinase, 20 mM KCl, 50 μM carboxyatractyloside, 50 μM Ap5A, 1 μM FCCP, and 50 μM [^{3}H]ATP. At the times shown, the mixtures were filtered twice through centrifuge columns. In the experiment 2, noncontrolled particles were loaded with [^{3}H]ADP by incubating 0.5 mg of particles in 0.1 mL of 200 mM sucrose, 10 mM $MgCl_2$, 20 mM Tris–acetate (pH 7.4), 20 mM KCl, 50 μM carboxyatractyloside, 50 μM Ap5A, 1 μM FCCP, 2 mM glucose, and 50 μM [^{3}H]ADP. After 10-min incubation the mixture was filtered once. Five filtrates were pooled and a sample was withdrawn to determine [^{3}H]ADP bound; this was zero time in experiment 2. To the rest, 10 mM Tris–acetate (pH 7.4), 10 mM $MgCl_2$, 0.1 mM ATP, 10 mM phosphoenolpyruvate, and 2 units of pyruvate kinase were added. At the indicated times, samples were withdrawn and filtered through centrifuge columns. [^{3}H]ADP used for experiment 2 was preincubated at a concentration of 0.5 mM, in the presence of 100 mM $MgCl_2$, 20 mM glucose, 20 mM Tris–acetate (pH 7.4), and 15 units of hexokinase for a few minutes to allow conversion of possible contaminating ATP; afterward, the mixture was placed in boiling water for 15 s to denature hexokinase. After centrifugation the mixture was used to make the incubation solution for the binding experiment.

Figure 5). However, the analysis of the ATPase activity was measured by pyruvate formation at times shorter than 30 s, with a concentration of particles about 200 times higher than in the spectrophotometric method, showed that in the first 5 s of reaction the specific activity was more than 2-fold higher than that at the steady state. This was allowed by a decrease which in less than 30 s reached the steady-state value.[2] These data suggest that the rapid inhibition of ATP hydrolysis could be a consequence of ADP binding to F_1. To test this possibility, the amount of [^{3}H]ADN bound to F_1 noncontrolled particles incubated with [^{3}H]ATP and excess pyruvate kinase and phosphoenolpyruvate was determined. It was found that 1.16 mol of [^{3}H]ADN bound to F_1 of noncontrolled particles in the first seconds of incubation (Table III, Experiment 1). HPLC analysis of the bound [^{3}H]ADN at 20 s of incubation showed that about 80% corresponded to ADP, yielding a ratio of approximately 0.9 mol of [^{3}H]ADP/mol of particulate F_1. Apparently there was a correlation between the appearance of [^{3}H]ADP in the enzyme and the inhibition of hydrolysis (compare Figure 5 and experiment 1 of Table III). Even though the media contained excess pyruvate kinase and phosphoenolpyruvate, it cannot be excluded that binding of [^{3}H]ADP occurred from medium [^{3}H]ADP that was not trapped.

Particles preincubated with ADP bind 1 mol of ADP/mol of F_1 which is inhibitory of hydrolysis (Figure 4B). Thus it was of interest to study if this inhibitory ADP is released during ATP hydrolysis. Accordingly, particles loaded with [^{3}H]ADP were allowed to hydrolyze ATP for several minutes, and the amount of [^{3}H]ADP that remained bound to F_1 was

[2] Even though pyruvate kinase was added at high concentrations, it was important to ascertain if pyruvate kinase could effectively trap ADP formed from hydrolysis. In the experimental media of Figure 5, it was found that in less than 5 s more than 400 nmol of ADP could be trapped by the amount of pyruvate kinase employed. Also, it was found that, within this time, ADP at a concentration below 20 μM was completely trapped.

estimated. It was found that, after several thousand catalytic cycles, only a small portion of [^{3}H]ADP was lost (Table III, experiment 2).

Taken together, the data of Figures 4 and 5 and Table III indicate that particulate F_1 has the capacity to rapidly bind one inhibitory ADP that may derive from ATP hydrolysis or medium ADP. Moreover, the data are consistent with a mechanism in which ADP at this binding site is inhibitory and exists in a steady state during stead-state hydrolysis. Finally, it should be noted that maximal rates of ATP hydrolysis could only be observed in times in which ADP derived from ATP hydrolysis (or from preincubation with ADP) was not bound to the enzyme.

Discussion

Different Mechanisms of Regulation of the Mitochondrial ATPase. An interesting aspect of the mitochondrial ATP synthase complex is the regulation of its hydrolytic activity. In contrat to the chloroplast ATP synthase complex, in which the hydrolytic activity is latent [for review see Shavit (1980)], the mitochondrial ATPase activity seems to be expressed to different degrees that depend on the type of preparation employed (Horstman & Racker, 1970; Van de Stadt et al., 1973; Klein et al., 1982; Beltrán et al., 1984). There are at least three recognized poinsts of control of this activity. One is the control of hydrolytic activity exerted by the electrochemical H^+ gradient in the coupled system as exemplified by the data in Table I. A second one is the inhibition of the ATPase activity of either soluble or particulate F_1-ATPase by the natural inhibitor protein (Pullman & Monroy, 1963). A third factor is the inhibiting effect of ADP on the enzyme. We now observe that the hydrolytic activity of coupled noncontrolled particles was inhibited by preincubation with ADP (Figures 2, 3, and 5) but that inhibition was higher when the particles were preincubated with ADP plus FCCP. Most likely this is due to the protonophoric action of FCCP, since 1799 (an uncoupler with a different chemical structure) exhibited the same effect (not shown).

It is important to note that the effect of uncouplers on the inhibition produced by ADP was observed in particles in which formation of $\Delta\tilde{\mu}_{H^+}$ was not induced (Figure 2), and in which no membrane potential was detected with oxonol. Although it is possible that the magnitude of the membrane potential was too low to be detected by oxonol, it is noteworthy that, in chloroplasts, Bar-Zvi and Shavit (1982) observed that the binding of ADP to CF_1 was favored by the presence of an uncoupler, even though the formation of $\Delta\tilde{\mu}_{H^+}$ was not provoked. These overall data suggest that the dissipation by an uncoupler of a localized proton difference or a small difficult to detect membrane potential potentiates the inhibitory action of ADP. The exact mechanism involved in the facilitation of the inhibitory effect of ADP by uncouplers remains to be established.

Half-maximal inhibition by NDP in the presence of uncouplers was obtained with 10 μM ADP or with 0.5 mM IDP. In parallel, it was observed that inhibition correlated in time with the binding of approximately 1 mol of ADP/mol of ATPase and that half-maximal binding took place with μM ADP. In agreement with these results, di Pietro et al. (1980) observed a $K_{0.5}$ of 14 μM for ADP for inhibition of hydrolytic activity of soluble F_1-ATPase, preincubated with ADP, the inhibition of hydrolysis being due to the binding of one ADP per enzyme. In this regard it is relevant that Vasilyeva et al. (1980, 1982) reported that release of the inhibiting effect of ADP can be achieved, but after prolonged incubation with pyruvate kinase and phosphoenolpyruvate. Recently, Kironde and Cross (1986) described that bovine heart F_1 incubated with Mg-ATP results in the binding of 1 ADP per enzyme that is not released during subsequent hydrolytic cycles; however, according to a statement of the authors (Kironde & Cross, 1986), it did not affect the hydrolytic rates. In our conditions in which the change of the rates in the first seconds of reaction were measured, we found that the binding of one ADP that arises during the course of ATP hydrolysis controls the activity of the enzyme (Figure 5). Although the binding of this ADP took place in the presence of a large excess of an ADP trap, it cannot be rigorously excluded that binding could have occurred from medium ADP. In fact, the latter possibility may be substantiated by the observation that this bound inhibitory ADP remains bound after several thousand catalytic cycles, which suggests that this ADP is not a catalytic site. Nevertheless, the data indicate that, at the steady state of ATP hydrolysis, F_1-ATPase would always be controlled by bound ADP, notwithstanding the presence of an ADP trap in the media.

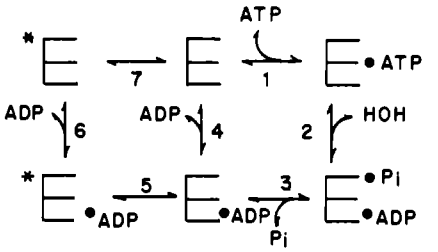

FIGURE 6: Model for the effect of bound ADP on ATP hydrolysis by F_1. It is visualized that F_1 can exist in two states, and E and *E; the latter is a form of low activity. For further details see text.

Scheme of Reactions. Our results can be explained through the scheme of reactions depicted in Figure 6. In this scheme, E and *E represent enzymes with a high and low activity, respectively. In the presence of ATP hydrolysis, after the binding of ATP to the E form (step 1), ATP is cleaved into ADP and P_i (step 2). The cycle is completed with the release of P_i and ADP (steps 3 and 4). In the absence of ATP and ADP, an equilibrium exists between the E and *E forms of the enzyme. Upon onset of hydrolysis, the equilibrium would be initially displaced to the various forms of the enzyme. During the pre-steady-state hydrolysis, some of the E·ADP forms could give rise to *E·ADP progressively (step 5) and the operation of steps 6 and 7 of the catalytic cycle. The true steady state would be established when the concentration of all intermediates is constant. If reaction 4 were faster than reaction 5, the pre steady state would be characterized by a high ATPase activity. According to the model, the high ATPase activity attained in the first seconds of reaction (Figures 1B,C and 5) is due to this pre steady state.

It was observed here that the ATPase activity of noncontrolled particles diminished upon binding of 1 ADP per enzyme. This suggests that the site that binds ADP in the *E form is regulatory and noncatalytic, particularly if it is considered that binding can occur from medium ADP and that after several thousand catalytic cycles only a small portion of the bound ADP was released, notwithstanding the presence of pyruvate kinase. Thus in the scheme, the existence of the enzyme in the *E·ADP form would poise the system at relatively low hydrolytic rates. Nevertheless, the finding that binding of this ADP occurred during hydrolysis of ATP in the presence of excess pyruvate kinase and phosphoenolpyruvate may suggest that, in the initial catalytic cycles, binding could have occurred at a catalytic site that subsequently would turn over at a rate lower than the other catalytic sites; this would bring about a diminution of the rate of hydrolysis. However, in the latter

experimental system, it cannot be excluded that binding of ADP from medium ADP could have taken place. It is pointed out that these mechanisms assume the existence of a homogeneous enzyme population.

Regardless of the mechanism involved, once ADP is bound at this site, the equilibrium of the enzyme forms would include, besides the E and *E, the E·ADP and *E·ADP forms. Experimentally, in particles preincubated with ADP (Figure 3) after the addition of ATP, we observed a low rate of ATP hydrolysis from the first seconds of reaction (compared to the enzyme preincubated in the absence of ADP).

The addition of FCCP together with ADP in the preincubation medium promoted a maximal decrease in the ATPase activity (Figures 2 and 3). The fact that this activity was constantly low from the first seconds indicates that, in this condition, the steady state was reached before 5 s of reaction. Therefore, in the model, it is suggested that uncouplers displace the equilibrium toward the low-activity forms of the enzyme through step 5 and/or step 7.

Registry No. ATPase, 9000-83-3; ADP, 58-64-0; IDP, 86-04-4.

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