

"Hysteretic" Behavior and Nucleotide Binding Sites of Pig Heart Mitochondrial F₁ Adenosine 5'-Triphosphatase[†]

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ABSTRACT: Preincubation of F₁ adenosine 5'-triphosphatase (F₁) from pig heart mitochondria with adenosine 5'-diphosphate (ADP) induces an inhibition which progressively develops in the presence of MgATP. This behavior is characteristic of hysteretic enzymes according to Frieden [Frieden, C. (1979) *Annu. Rev. Biochem.* 48, 471-489]. The presence of Mg²⁺ in the preincubation medium increases the affinity for ADP. The inhibition can be prevented or slowly reversed when ATP is added to the preincubation medium in the absence of Mg²⁺, but it is not reversible by simple dilution. Preincubated with [¹⁴C]ADP, nucleotide-depleted F₁ binds 3 mol/mol as measured by gel filtration (in the presence of Mg²⁺ and the absence of nucleotides) while F₁ containing 1.8 mol of tightly bound ADP/mol only binds about 2 mol of [¹⁴C]-ADP. In both cases dimethyl sulfoxide prevents the binding of 1 mol of ADP/mol of enzyme but does not modify the inhibition. With nucleotide-depleted F₁, the binding of the

first mole of ADP (that with a higher affinity) is not correlated with enzymatic inhibition. With F₁, although ~1 mol of ADP is sufficient to produce the inhibition in the presence of dimethyl sulfoxide, the binding of 2 mol of ADP occurs simultaneously with the inhibition. The inhibition and the related binding of ADP are reversed by (NH₄)₂SO₄ precipitation suggesting that this inhibitory ADP site and tightly bound sites are distinct. The inhibitory site also appears to be distinct from the catalytic site since the binding of radioactive ADP is neither prevented by inhibitors of the enzyme activity (dicyclohexylcarbodiimide, 4-chloro-7-nitrobenzofurazan) nor released when the enzyme is turning over in the presence of MgATP. The results suggest that Mg²⁺ provokes entrapping of ADP into a regulatory site different from the catalytic site and not directly accessible to the external medium; then the addition of MgATP induces a conformational change that finally leads to the hysteretic inhibition.

Results from this laboratory (Godinot et al., 1975; Di Pietro et al., 1979) and from others (Hilborn & Hammes, 1973; Ebel & Lardy, 1975; Schuster et al., 1975; Garrett & Penefsky, 1975b; Pedersen, 1975; Leimgruber & Senior, 1976; Recktenwald & Hess, 1977; Harris et al., 1978; Slater et al., 1979) have provided support for the view that regulatory nucleotide-binding sites, distinct from catalytic sites, are present in mitochondrial F₁ adenosine 5'-triphosphatase (F₁).¹ Numerous experimental data suggest that the catalytic sites are located on the β subunits (Budker et al., 1977; Verschoor et al., 1977; Wagenvoort et al., 1977; Esch & Allison, 1978; Pougeois et al., 1979; Slater et al., 1979). The recent use of nucleotide analogues bearing azido groups (Russell et al., 1976) has shed light on nucleotide-site location. Azido derivatives of ATP (Cosson & Guillory, 1979) or ADP (Lunardi et al., 1977; Wagenvoort et al., 1979) can be bound both on the α and β subunits; 8-azido-ADP mainly reacts with the α subunit in the presence of Mg²⁺ (Wagenvoort et al., 1979).

The purpose of our work was to study the regulatory role played by the binding of ADP under conditions in which ADP mainly binds to the α subunit according to the results of Wagenvoort et al. (1979). It is shown that after preincubation of F₁-ATPase in the presence of low concentrations of ADP plus or minus Mg²⁺, the addition of MgATP progressively provokes a decrease of the enzyme activity leading to a stable inhibited state. This inhibition is due to the binding of ADP on the enzyme not rapidly reversible by simple dilution or gel filtration in the presence of magnesium but reversed by ammonium sulfate precipitation. Studies made in the presence or absence of dimethyl sulfoxide show that the binding of ~1 mol of MgADP provokes the inhibition; the bound ADP is not released when the enzyme is turning over in the presence of

MgATP. The same conclusions are reached when using F₁ or nucleotide-depleted F₁. It is proposed that ADP regulates the ATPase activity by binding to the ADP inhibitory site(s) distinct from the catalytic site.

Experimental Procedures

Materials. Nucleotides were purchased from Boehringer Mannheim; their purity was monitored by high-pressure liquid chromatography as described (Di Pietro et al., 1979). Labeled nucleotides were obtained from the Radiochemical Center, Amersham, England, as [U-¹⁴C]ADP, 510 mCi/mmol, and [U-¹⁴C]ATP, 520 mCi/mmol. All other reagents were of the highest purity commercially available.

Enzyme Preparation. Pig heart mitochondria were obtained as previously described (Gautheron et al., 1964). The mitochondrial F₁-ATPase was purified by two methods. The first method, derived from that of Senior & Brooks (1970) as reported earlier (Di Pietro et al., 1975, 1979), provided a preparation of F₁-ATPase exhibiting a specific activity of 90-110 units/mg of protein (1 unit = 1 μ mol of ATP hydrolyzed per min). This enzyme was stored in 40 mM Tris-H₂SO₄, 1 mM EDTA, and 2 mM ATP, pH 7.5, as an ammonium sulfate suspension at 0-4 °C; it contained 1.8 \pm 0.2 mol of ADP and 0.3 \pm 0.03 mol of ATP/mol (Penin et al., 1979) and will be referred to as F₁. The second method (Penin et al., 1979) combined chloroform extraction (Beechey et al.,

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¹ Abbreviations used: Me₂SO, dimethyl sulfoxide; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; DCCD, dicyclohexylcarbodiimide; ATPase, adenosine 5'-triphosphatase; F₁, pig heart mitochondrial F₁-ATPase prepared according to the procedure of Senior & Brooks (1970) as modified by Di Pietro et al. (1975, 1979); nucleotide-depleted F₁, pig heart mitochondrial F₁-ATPase prepared according to the methods described by Beechey et al. (1975) and Garrett & Penefsky (1975a) as described (Penin et al., 1979); arylazido-ADP, N-4-azido-2-nitrophenyl- γ -aminobutyryl-adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; PEI, poly(ethylenimine).

1975) and gel filtration in the presence of 50% glycerol (Garrett & Penefsky, 1975a); the obtained enzyme, virtually devoid of tightly bound nucleotides (0.17 ± 0.18 mol of ADP and 0.27 ± 0.04 mol of ATP/mol), had a specific activity of 120–140 units/mg of protein; it was kept at -80°C in 100 mM Tris- H_2SO_4 buffer containing 5 mM EDTA and 50% glycerol, pH 8.0, and will be called nucleotide-depleted F_1 .

Whenever used, F_1 was freed from the nucleotides present in the medium by the following procedure: an aliquot of the stock solution was centrifuged for 5 min at 9000g in an "Eppendorf Zentrifuge 3200"; the pellet was dissolved in 40 mM Tris- H_2SO_4 buffer containing 1 mM EDTA at pH 7.5; saturated ammonium sulfate was added up to a concentration of 2.6 M, and the suspension was centrifuged again. After four identical washings, the enzyme was dissolved in the above buffer unless otherwise indicated.

The β subunit was prepared as previously described by Penin et al. (1979). The protein content of the enzyme solutions was estimated either by the Lowry procedure (Lowry et al., 1951), taking bovine serum albumin as the standard, or by the Bradford method (Bradford, 1976) using, as a reference, a solution of F_1 previously standardized with the Lowry procedure. The molecular weight of F_1 was taken as 380 000 (Di Pietro et al., 1975).

Enzyme Activities. The ATPase activity was determined in the presence of magnesium by a spectrophotometric assay with an ATP-regenerating system or, in some cases, by inorganic phosphate measurement as described previously (Di Pietro et al., 1975). In the absence of magnesium, the rate of ATP hydrolysis was estimated by incubating the enzyme (1 mg of protein/mL) in the presence of 3 mM EDTA, 80 mM Tris- H_2SO_4 , pH 8.0, 1 mM $[\text{U-}^{14}\text{C}]\text{ATP}$ (14 mCi/mmol), and 5% dimethyl sulfoxide for several hours. From time to time, aliquots were removed and spotted on thin-layer chromatographic plastic sheets precoated with poly(ethylenimine)-cellulose F (Merck). Two nanomoles of unlabeled ATP, ADP, and AMP was added to each aliquot. Following ascending chromatography in 0.7 M LiCl, the nucleotides were located under UV light at 254 nm, the spots were scraped and counted for radioactivity in 10 mL of toluene containing 4 g of diphenyloxazole and 0.125 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per L. The decrease in radioactivity of the ATP spot was parallel to the increased radioactivity of the ADP spot. The initial rate of ATP hydrolysis could then be estimated. The activity of adenylate kinase (EC 2.7.4.3) was estimated by the same method except that 1 mM $[\text{U-}^{14}\text{C}]\text{ADP}$ (11 mCi/mmol) replaced $[\text{U-}^{14}\text{C}]\text{ATP}$ and that 1 mM MgSO_4 replaced EDTA. In this case, the activity was measured by the appearance of radioactivity into AMP and its disappearance from ADP.

Conditions of Preincubation of Enzyme with ADP for Kinetics and Binding Measurements. To study the inhibition of ATPase induced by ADP and the binding of $[\text{U-}^{14}\text{C}]\text{ADP}$, we incubated the enzyme (0.5 mg of protein/mL or 1.5 mg/mL in dialysis experiments) at 30°C , in 80 mM Tris- H_2SO_4 , pH 8.0, 15% glycerol, with or without 7.5% dimethyl sulfoxide, and 1 mM EDTA or 1.5 mM MgCl_2 at various ADP concentrations. From time to time, 1–5- μL aliquots were transferred into the spectrophotometer cuvette containing all the ingredients necessary for measuring the ATPase activity. Approximately 5 s were necessary before beginning the recordings of NADH disappearance after introduction of the sample.

To determine the amount of bound ADP, we added $[\text{U-}^{14}\text{C}]\text{ADP}$ (0.5×10^6 – 2×10^6 dpm) and determined the bound

ADP following equilibrium dialysis or gel filtration. Dialysis was performed in a Dianorm apparatus equipped with 10 half-cells separated by a dialysis membrane (2-cm diameter) and the solution continuously stirred by rotation with the help of a small motor. Each cell contained 0.125 mL of reaction mixture. In this system, the equilibrium between the two half-cells could be reached within 45 min at 20°C in the absence of enzyme. The experiments were stopped after 3 h when the enzyme was present.

In gel filtration experiments, the enzyme was preincubated for 15 or 60 min in the presence of 0.5 mM $[\text{U-}^{14}\text{C}]\text{ADP}$ unless otherwise indicated; then a sample of 100 μL was layered on the top of a column (1.3 \times 35 cm) of Sephadex G-50 (medium) equilibrated at pH 8.0 in 100 mM Tris- H_2SO_4 , in the absence or in the presence of 7.5% dimethyl sulfoxide with or without 1.5 mM MgCl_2 . The enzyme was eluted in 10 min with the same buffer; 0.8-mL fractions were collected. Alternatively, the bound ADP was separated from the free ADP by the filtration-centrifugation method described by Penefsky (1977), in the same medium used for gel filtration. Enzyme activity and protein content were determined as described above. Radioactivity was measured in a scintillation cocktail made of 50% Triton X-114, 50% toluene, and 4 g of 2,5-diphenyloxazole/L.

Use of Inhibitors of ATPase Activity. Nbf-Cl (0.1 mM) was added to the enzyme under the same conditions as those used in the preincubation of the enzyme with ADP (see above) but in the absence of ADP. Aliquots of the incubation medium were used to assay enzymatic activity spectrophotometrically. When an inhibition of at least 75% was reached, 0.5 mM $[\text{U-}^{14}\text{C}]\text{ADP}$ was added together with 1.5 mM MgCl_2 , and, 1 h later, the enzyme was subjected to Sephadex G-50 column filtration to determine the quantity of bound ADP.

DCCD dissolved in methanol was incubated with the enzyme at a concentration of 2 mM under the above conditions except that ADP and MgCl_2 were omitted and that the pH was 7.0. When the inhibition reached the indicated value, unbound DCCD was eliminated by the filtration-centrifugation method described by Penefsky (1977) according to Pougeois et al. (1979). The eluate was incubated with 0.5 mM ADP and 1.5 mM MgCl_2 for 1 h and then subjected to filtration through a Sephadex G-50 column equilibrated with 100 mM Tris- H_2SO_4 and 1.5 mM MgSO_4 , pH 7.0. Indeed, filtration at pH 8.0 partly reversed the DCCD-induced inhibition. In the absence of DCCD treatment, ADP binding was identical when the filtration was carried out at pH 7.0 or 8.0.

ATP and ADP Estimation. ATP was determined after denaturation of F_1 -ATPase by photon counting using the firefly luciferase assay as described (Penin et al., 1979). ADP was measured by the same method after complete conversion into ATP by incubation for 30 min at 30°C in the presence of 0.7 mM phosphoenolpyruvate, 5 mM MgSO_4 , 5 mM KCl, 20 mM Tris- H_2SO_4 , pH 7.8, and 50 μg of pyruvate kinase (100- μL final volume).

Calculations. Results are presented as the mean of several experiments followed by the standard error of that mean.

Results

Influence of Preincubation of F_1 with ADP on the Time Course of ATP Hydrolysis. The amount of ADP or P_i produced during ATP hydrolysis increased linearly with the reaction time when the enzyme was preincubated in the absence of nucleotides (Figure 1, no ADP). On the other hand, when the enzyme had been preincubated for 10 min in the presence of 0.5 mM ADP and 1.5 mM MgCl_2 , the kinetics were bi-

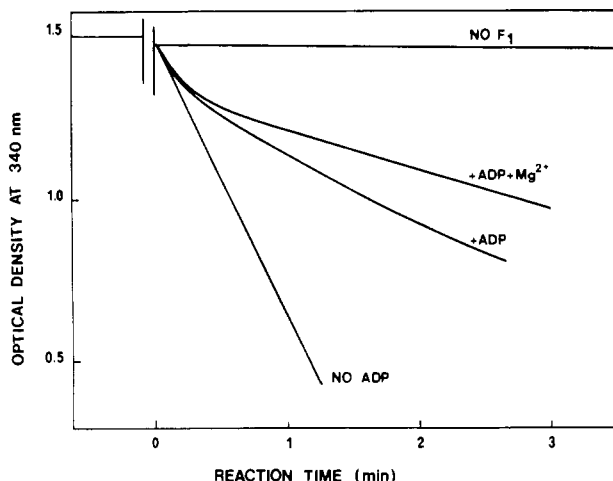


FIGURE 1: Recordings of the rate of ATP hydrolysis after preincubation of F_1 in the presence or absence of ADP and Mg^{2+} . F_1 (0.5 mg of protein/mL) was preincubated in 80 mM Tris- H_2SO_4 , \pm 15% glycerol, and \pm 5% dimethyl sulfoxide, pH 8.0, at 30 °C, in the presence of either 0.5 mM ADP and 1 mM EDTA or 0.5 mM ADP and 1.5 mM $MgCl_2$; the final volume was 100 μ L. After a 10-min preincubation, 2- μ L aliquots were removed and diluted 310 times into the standard ATPase assay medium containing 3.3 mM ATP, 3.3 mM $MgCl_2$, and a regenerating system (phosphoenol pyruvate, pyruvate kinase, lactic dehydrogenase, and NADH) as previously described (Di Pietro et al., 1975). The ATPase activity was followed spectrophotometrically as soon as possible (5–10 s after the addition of F_1). Controls were made by omitting ADP (no ADP) or F_1 (no F_1) during preincubation.

phasic: during the first seconds, the ATP hydrolysis started at a rate identical with the control but progressively diminished until a constant inhibited rate was reached. This steady-state rate was always reached within 1 min of initiation of the reaction, whatever the ADP concentration was during preincubation. If Mg^{2+} was omitted from the preincubation medium, the inhibition during the course of Mg ATP hydrolysis was obtained more slowly, especially at low ADP concentrations, and continued to slightly increase with time.

In these experiments, the reaction was always initiated by taking samples of 2 μ L from the preincubation medium (containing the enzyme) and introducing them into the reaction mixture (620 μ L). Under these conditions, the ADP present in the preincubation was diluted more than 300 times and could not interfere with the reaction. Indeed, for the controls run in the absence of F_1 (Figure 1, no F_1), the ADP from the incubation mixture was transformed before any recording could be made; this can be explained by the very high activity of the pyruvate kinase present. It will be seen that the inhibition persists after elimination of free ADP by gel filtration. The same type of biphasic curves were obtained with F_1 and nucleotide-depleted F_1 ; the phenomenon was independent of the presence of dimethyl sulfoxide (7.5%) and glycerol (15%).

Influence of Conditions of Preincubation on the Rate of ATP Hydrolysis during the Steady-State Phase of the Reaction. When considering variations of the stable inhibited rate (obtained after 1 or 2 min) of ATP hydrolysis following preincubation with ADP in the presence or absence of Mg^{2+} , the following observations, illustrated by Figure 2, can be made. (1) The extent of inhibition obtained in this steady-state phase depends on the duration of preincubation of the enzyme with ADP. (2) A plateau is reached after about a 2-min preincubation at high ADP concentration and a 10-min preincubation at the lowest ADP concentration at which inhibition is observed. (3) The maximum inhibition obtained increases with ADP concentration.

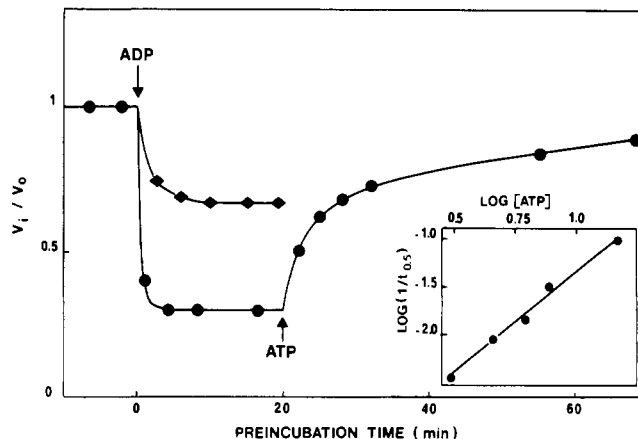


FIGURE 2: Time course of ADP-induced inhibition of ATPase activity and reversion by ATP. The enzyme (F_1 here) was preincubated as described in Figure 1 in the presence of 0.1 mM (\diamond) or 1 mM (\bullet) ADP and 1 mM EDTA. Aliquots were withdrawn at intervals and introduced into the spectrophotometer cuvette. The inhibited rate v_1 was measured after 2 min of reaction for a period of 1 min; under these conditions the recordings were approximately linear. A control was made in the absence of ADP for the determination of v_0 . To study the reversal of inhibition, we preincubated the enzyme with or without 1 mM ADP, and at 20 min we added 15 mM ATP and measured the rates v_1 or v_0 on aliquots as a function of time. (Insert) Influence of ATP concentration on the rate of reactivation. The reversion of inhibition was studied at ATP concentrations ranging from 1.5 to 15 mM. the time ($t_{0.5}$) necessary to observe 50% reactivation was measured, and the log of ($1/t_{0.5}$) was plotted against the log of the ATP concentration according to the method described by Levy et al. (1963).

If $MgCl_2$ (1.5 mM) is added to the preincubation medium after the plateau has been reached, the inhibition further increases progressively until a new inhibitory level is reached (not shown). When $MgCl_2$ is present before the addition of ADP, the maximal inhibition obtained with a given ADP concentration is greater. The presence of 10 mM P_i or 5 mM AMP does not affect the inhibition. When ATP was added to the preincubation medium before ADP, in the absence of Mg^{2+} to avoid hydrolysis, the maximal inhibition was diminished. If ATP was added alone, there was no change in the rate of ATP hydrolysis. If ATP was added 20 min after ADP, the activity was progressively and completely recovered (Figure 2). F_1 and nucleotide-depleted F_1 behaved similarly under these conditions. Whatever the ATP concentration, a complete reactivation was observed. Similar observations have been made by Harris et al. (1978), but it is shown here that the rate of reversal of the inhibition is dependent on the ATP concentration: when the log of $1/t_{0.5}$ ($t_{0.5}$ = time necessary to obtain 50% reversion) is plotted against the log of ATP concentration, a straight line is observed. A slope of 2.1 is calculated with the nucleotide-depleted F_1 (insert of Figure 2), and similar values were obtained with F_1 . This suggests that at least 2 mol of ATP should be bound per mol of enzyme in order to observe the reversion. Moreover, the biphasic kinetics of the ATP hydrolysis progressively became linear as the ADP inhibition was reversed by ATP (not shown).

Figure 3 shows the variations of the maximal inhibition observed as a function of the ADP concentration in the preincubation medium. The concentration for 50% inhibition was 14 μ M for nucleotide-depleted F_1 and 20 μ M for F_1 in the presence of 1.5 mM $MgCl_2$. In the absence of $MgCl_2$, the ADP concentration for 50% inhibition was 4–5-fold higher (not shown). Experimentally, a complete inhibition was never observed; F_1 and nucleotide-depleted F_1 showed the same residual activity although the initial activity of nucleotide-depleted F_1 was higher than that of F_1 . Even when the ADP

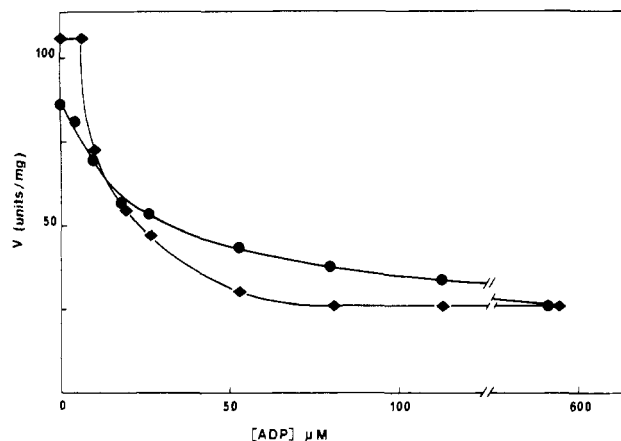


FIGURE 3: ADP-induced inhibition as a function of ADP concentration. F_1 (●); or nucleotide-depleted F_1 (◆) was preincubated under the same conditions as described in Figure 2 except that ADP concentration varied and 1.5 mM $MgCl_2$ replaced EDTA. After a 10-min preincubation the ATPase activity was measured as described in Figure 2.

concentration was increased to 5 mM, the same residual activity was observed indicating that a complete inhibition is never obtained. At very low ADP concentrations, nucleotide-depleted F_1 was not inhibited while F_1 was sensitive at the lowest ADP concentration tested (4 μM). The differences observed between F_1 and nucleotide-depleted F_1 cannot be explained by different incubation conditions: indeed, before F_1 was used free nucleotides were removed by four successive ammonium sulfate precipitations, and the preincubation was made in the presence of the same amount of glycerol as that present in assays made with nucleotide-depleted F_1 . In some experiments, F_1 had only been washed once; its specific activity was somewhat lower than that for the F_1 washed 4 times, and the maximal inhibition was limited to 50–60%. It cannot be excluded that ATP closely associated with the enzyme could partly prevent the ADP-induced inhibition since ATP can release the inhibition (see Figure 2).

Binding of ADP. The fact that the enzyme preincubated with ADP is still inhibited after dilution in the reaction mixture indicates that either ADP remains strongly associated with the enzyme or ADP induces a stable conformational change, or else there is a combination of the two possibilities. To decide between these alternatives, we determined the amount of ADP remaining bound to the enzyme after dilution by gel filtration. The enzyme was preincubated with radioactive nucleotide under different conditions and passed through a Sephadex G-50 column, in the absence of added nucleotide.

Figure 4 shows that the amount of [^{14}C]ADP remaining bound on F_1 or nucleotide-depleted F_1 after gel filtration in the presence of 1.5 mM $MgCl_2$ varied as a function of the ADP concentration present during the preincubation; a maximal number of 2.2 ± 0.2 mol of ADP remained bound per mol of F_1 and 3.0 ± 0.2 to nucleotide-depleted F_1 . The insert of Figure 4 correlates the binding of ADP and the observed inhibition: 1 mol of ADP is bound per mol of nucleotide-depleted F_1 without affecting enzymatic activity while further binding of another 2 mol of ADP is accompanied by a decrease in activity. In contrast, with F_1 this inhibition is directly proportional to the amount of bound ADP with no apparent discrimination of different binding for the 2 mol of ADP. It should be emphasized that extrapolation to 100% inhibition is not valid since a complete inhibition is never reached, as mentioned above. The ADP concentration for the apparent half-saturation is respectively 8 and 14 μM for nu-

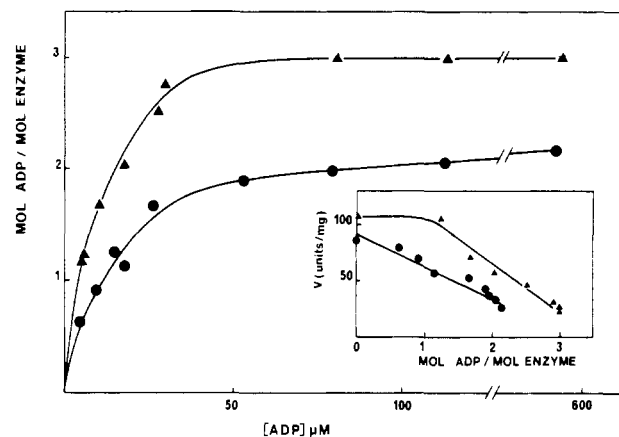


FIGURE 4: Variations of the amount of [^{14}C]ADP remaining bound to the enzyme after gel filtration as a function of ADP concentration. F_1 (●) or nucleotide-depleted F_1 (▲) was preincubated for 20 min in the presence of the indicated concentrations of [^{14}C]ADP; the radioactivity remaining bound after filtration–centrifugation through a Sephadex G-50 column equilibrated in the presence of 1.5 mM $MgCl_2$ and in the absence of added ADP was determined as described under Experimental Procedures. (Insert) Relationship between the amount of ADP remaining bound and the induced inhibition. The residual ATPase activities obtained in Figure 3 for different ADP concentrations were plotted against the amounts of [^{14}C]ADP remaining bound (obtained in this Figure) when the same ADP concentrations were added.

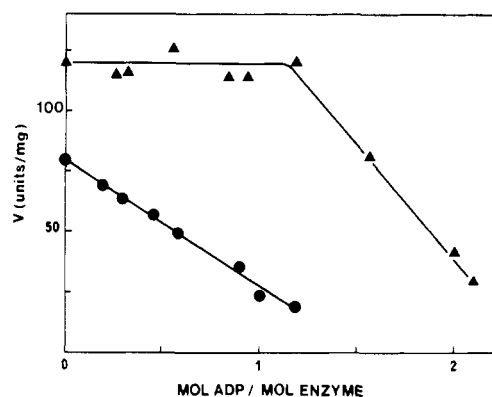


FIGURE 5: Relationship between the ADP-binding and the induced inhibition in the presence of Me_2SO . F_1 (●) or nucleotide-depleted F_1 (▲) was submitted to the same conditions as described in Figures 3 and 4 except that 7.5% Me_2SO was added during preincubation and gel filtration.

cleotide-depleted F_1 and F_1 (Figure 4).

When Me_2SO was added to the assay mixture to improve the stability of the enzyme, especially at low protein concentration and in the presence of 1.5 mM $MgCl_2$, the extent of the inhibition was never modified while the amount of ADP bound decreased by ~ 1 mol/mol of enzyme as compared to assays conducted in the absence of Me_2SO (Table I).

Comparison of Figure 5 and the insert of Figure 4 shows that the maximal inhibition is observed when 1.2 mol of ADP is bound per mol of F_1 in the presence of Me_2SO instead of 2.2 in the absence of Me_2SO . With the nucleotide-depleted F_1 the binding of 1.2 mol of ADP/mol does not affect the ATPase activity in both cases, the inhibition being correlated with the additional binding of ~ 1 mol in the presence of Me_2SO or 2 mol/mol in the absence of Me_2SO .

Table I also shows that preincubation of the enzyme with ADP or ATP followed by gel filtration in the absence of magnesium leads to the binding of very low amounts of nucleotide; the ADP-induced inhibition of ATPase activity is suppressed by the gel filtration. The absence of magnesium

Table I: Nucleotides Remaining Bound to Enzyme after Gel Filtration in the Absence of Nucleotide^a

enzyme preparation	preincubation conditions	MgCl ₂ in gel filtration (mM)	bound ¹⁴ C-labeled nucleotide (mol/mol of enzyme)	% inhibition of ATPase activity	
				before gel filtration	after gel filtration
F_1	MgADP, 1 h	1.5	2.2 ± 0.2	72	
nucleotide-depleted F_1	MgADP + Me ₂ SO, 1 h	1.5	1.2 ± 0.1	71	
	MgADP, 1 h	1.5	3.0 ± 0.2	77	75
	MgADP + Me ₂ SO, 1 h	1.5	2.2 ± 0.2	78	75
	ADP + Me ₂ SO, 1 h	0	0.13	55	0
	ATP + Me ₂ SO, 1 h	0	0.12	0	0
	ADP + Me ₂ SO, 1 h	1.5	2.05 ± 0.1	55	
	MgADP + Me ₂ SO, 1 h	0	1.3	78	15
	MgATP + Me ₂ SO, 1 h	1.5	2.2	78	76
	ADP + Me ₂ SO, 2 min	1.5	0.8	41	

^a The enzyme solution was preincubated in the presence of 0.5 mM radioactive ADP or ATP with or without MgCl₂; the gel filtration was performed in the absence of added nucleotide and with or without MgCl₂; when present, 7.5% Me₂SO was added during both preincubation and gel filtration. The amount of nucleotide remaining bound to the enzyme in the eluate was determined as described under Experimental Procedures.

only in the preincubation diminishes slightly the amount of ADP bound. The absence of magnesium only during the gel-filtration decreases significantly the amount of bound ADP. If [¹⁴C]ATP replaced ADP, very similar results were obtained; this could be expected since the preincubation medium contained so much enzyme activity that all the added ATP was transformed into ADP in <2 min in the presence of MgCl₂ as checked by thin-layer chromatography on PEI-cellulose. If the filtration was made only 2 min after the beginning of the incubation, the inhibition and the amount of bound ADP were lower; if the incubation lasted between 15 min and 1 h, there was no difference.

The binding of radioactive ADP could be partly reversed when, after gel-filtration, F_1 or nucleotide-depleted F_1 was precipitated with ammonium sulfate: after one or two precipitations, only 0.16 mol of ADP/mol of nucleotide-depleted F_1 and 0.08 mol/mol of F_1 remained bound when Me₂SO was present during the binding procedure; if Me₂SO was omitted, more radioactive ADP remained bound, about 1 mol/mol of nucleotide-depleted F_1 and 0.5 mol/mol of F_1 .

Since Harris (1977) reported that ammonium sulfate concentrations higher than 60% saturation might remove tightly bound nucleotides, such a possibility was checked under our conditions. The bound [¹⁴C]ADP was removed to the same extent when the ammonium sulfate concentration ranged from 50 to 70% saturation. After ammonium sulfate precipitation, the high specific enzymatic activity was recovered, and the biphasic ATPase kinetics were not observed.

If 4 mM MgATP was added to nucleotide-depleted F_1 previously preincubated with 0.5 mM [¹⁴C]ADP, 1.5 mM MgCl₂, and 7.5% Me₂SO and the mixture submitted to gel filtration 5 min later in the presence of 1.5 mM MgCl₂, the amount of bound [¹⁴C]ADP was not modified (2.1 mol/mol). Under these conditions, MgATP was completely hydrolyzed as checked by thin-layer chromatography on PEI-cellulose. This experiment shows that the hydrolysis of ATP at the active site did not drive [¹⁴C]ADP out of its sites. The cold ADP produced during the hydrolysis of ATP was not significantly exchanged with the previously bound [¹⁴C]ADP. A final ammonium sulfate precipitation permitted detachment of most of the radioactive ADP, as previously.

The total number of nucleotides present on F_1 after the binding experiment amounted to the bound [¹⁴C]ADP in addition to the tightly bound nucleotides already present. The ATP did not change (0.15 mol/mol of F_1) and the total ADP increased from 1.7 to 2.7 mol/mol under conditions where 1.1 mol of [¹⁴C]ADP was bound per mol (Me₂SO present). This

Table II: Influence of Cations on ADP Remaining Bound after Gel Filtration^a

cation	bound [¹⁴ C]ADP (mol/mol of nucleotide-depleted F_1)
none	0.13
Mg ²⁺	2.3
Ca ²⁺	0.3
Mn ²⁺	2.0
Co ²⁺	1.9
Zn ²⁺	2.1

^a Nucleotide-depleted F_1 was preincubated with 0.5 mM [¹⁴C]-ADP for 1 h in the presence of Me₂SO. The cations were added to a final concentration of 1.5 mM during both preincubation and gel filtration.

indicates that no significant exchange has occurred between the tightly bound and the radioactive bound ADP during the 30 min of the experiment.

After gel filtration (in the presence of MgCl₂) to remove the free ADP during the determination of bound radioactive ADP, the enzyme remained inhibited provided its activity was measured immediately. The inhibition patterns of F_1 or nucleotide-depleted F_1 as a function of ADP concentration were similar to those described in Figures 1 and 3 before gel filtration. When the enzyme eluted from the gel filtration was allowed to stand at room temperature for several hours, the activity measured on aliquots from time to time slowly increased and the initial rate of ATP hydrolysis progressively became linear. The bound [¹⁴C]ADP was slowly released as measured by a second gel filtration made from time to time after the end of the first gel filtration: a half-time of reversion of ~3 h has been obtained with F_1 preincubated in the presence of Me₂SO.

Influence of Various Treatments of F_1 on Binding. Inhibition and binding were not significantly modified if the pH was changed between 7.0 and 8.0. There was no binding of [¹⁴C]ADP on the isolated β subunit when assayed after gel filtration under the same conditions as those described for F_1 . When F_1 was denatured by cold treatment in ice until 96% of the activity had disappeared, only 0.12 mol of [¹⁴C]ADP was found to be bound per mol of F_1 .

Influence of the Presence of Various Cations. Table II shows that in addition to Mg²⁺, Mn²⁺, Co²⁺, and Zn²⁺ were able to ensure the binding of [¹⁴C]ADP, their efficiency being slightly lower than that of Mg²⁺. Ca²⁺ is very much less effective. The relative influence of cations on the binding of ADP can be compared to the rate of ATP hydrolysis in the

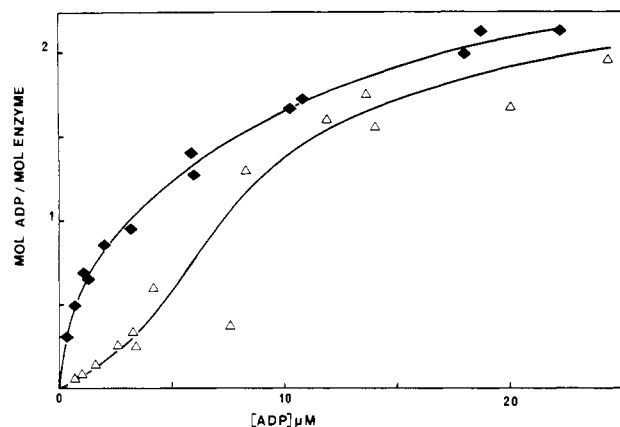


FIGURE 6: Binding of [^{14}C]ADP on nucleotide-depleted F_1 as measured by equilibrium dialysis in the presence of Me_2SO . Conditions were as described under Experimental Procedures. (Δ) No Mg^{2+} ; (\blacklozenge) 1.5 mM Mg^{2+} .

presence of the same cations (C. Van Herrewege, unpublished observations).

Effects of Various Inhibitors on ADP Binding. Pretreatment of both F_1 and nucleotide-depleted F_1 with 2 mM DCCD for 135 min resulted in an inhibition of ATPase activity of 82% but did not diminish the amount of bound [^{14}C]ADP. Pretreatment with 0.1 mM Nbf-Cl inducing an inhibition of 76 or 92% did not affect the binding. Pretreatment of nucleotide-depleted F_1 with 6.7 mM *p*-(chloromercuri)benzoate did not affect the ATPase activity, as measured by phosphate liberation under the conditions of the experiment, provided all calibration curves were made in the presence of *p*-(chloromercuri)benzoate. The binding of [^{14}C]ADP on *p*-(chloromercuri)benzoate-modified enzyme was not affected. The *p*-(chloromercuri)benzoate was diluted to 2.7 mM during the binding determination.

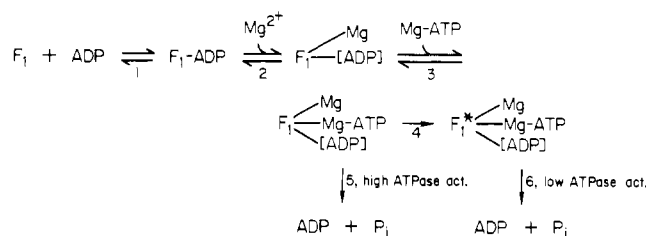
Studies of Binding of [^{14}C]ADP by Equilibrium Dialysis. The fact that part of the ADP bound to the enzyme was not removed by dilution means that this ADP is not in rapid equilibrium with the external medium. Under these conditions, the significance of experiments made by measuring ADP bound to the enzyme by equilibrium dialysis is limited. However, at low nucleotide concentrations, such experiments may give some information. Figure 6 shows that in the absence of magnesium and at ADP concentrations too low to observe biphasic kinetics and enzymatic inhibition, two sites of the nucleotide-depleted F_1 can be saturated with ADP. There is a strong positive cooperativity between these sites. In the presence of 1.5 mM magnesium, two kinds of sites seem to be accessible in the same range of concentration. The experiments done here cannot permit the distinction between two independent binding sites or sites interacting with negative cooperativity. It must be noticed that in the presence of magnesium, the upper ADP concentrations studied are already inhibitory.

Furthermore, it should be mentioned that the nucleotide-depleted F_1 used in this report had very low adenylate kinase activity ($0.93 \text{ nmol min}^{-1} (\text{mg of protein})^{-1}$). The ATPase activity measured in the absence of Mg^{2+} (see Experimental Procedures), $0.87 \text{ nmol min}^{-1} (\text{mg of protein})^{-1}$, was $\sim 140\,000$ -fold lower than that measured in the presence of added Mg^{2+} .

Discussion

The data presented in Figure 1 clearly justify the term of hysteretic inhibition. Indeed, according to Frieden (1979), "enzymes that show hysteretic behavior are defined as those

Scheme I



$F_1\text{-[ADP]} = \text{ADP entrapped binding site}$

that respond slowly (in terms of their kinetic behavior) to a change in ligand concentration, e.g., the addition of substrate to initiate the reaction". After preincubation with ADP, the enzyme can turn over at a rapid rate only for a very short time and within 1 min reaches a partly inactivated state. The ADP bound is not in equilibrium with the medium, since the inhibition takes place when the enzyme is diluted 300-fold in the reaction mixture containing MgATP and an ATP-regenerating system which does not allow any ADP to be present in the medium. However, when ATP is added after ADP in the absence of magnesium to the preincubation medium, the inactivation is slowly reversed with the time of reversion dependent on the ATP concentration. The inactivation is magnesium dependent. All the data can be fitted into the proposed Scheme I which includes a conformational transition of the enzyme from a high to a low ATPase activity.

Step 1 is a reversible binding of ADP; indeed, in the absence of Mg^{2+} , ADP can be released by gel filtration or ATP addition. In step 2, the addition of Mg^{2+} either during preincubation or in the reaction mixture entraps ADP in a specific site which is no longer in equilibrium with the external medium ADP. The rate-limiting step is relatively slow since a preincubation of 2–10 min is necessary to observe maximal binding and a concomitant inhibition. When ADP is preincubated in the absence of Mg^{2+} , the inhibition is established during the course of ATP hydrolysis more slowly than if Mg^{2+} is present during preincubation. In this case inhibition increases with time while a linear rate is obtained following a 1-min incubation in the presence of Mg^{2+} . Step 1 is easily reversed by gel filtration in the absence of magnesium. In step 3, the binding of the substrate MgATP which does not release the bound ADP allows two different effects. On the one hand, it provokes a slow transition of the enzyme toward a partially inactive form F_1^* (reaction 4); on the other hand, it initiates ATPase activity for both reactions 5 and 6. The return to the initial active form F_1 can be achieved by ammonium sulfate precipitation which releases the ADP from its entrapping binding site. This model is valid as well as for F_1 or nucleotide-depleted F_1 .

Many questions can be raised about this model. (1) How many moles of ADP are related to the inactivation process? The rate of reversion by ATP of the ADP-induced inhibition studied as a function of the ATP concentration in the absence of Me_2SO is consistent with the involvement of two nucleotide binding sites. During inhibition, 2 mol of ADP/mol of F_1 and 3 mol of ADP/mol nucleotide-depleted F_1 are bound. Addition of dimethyl sulfoxide prevents the binding of 1 mol of ADP/mol of either form of enzyme without affecting the extent of the inhibition. On the other hand, for nucleotide-depleted F_1 the binding of the first mol of ADP is not related to the inhibition (insert of Figure 4 and Figure 5). Therefore, the binding of ~ 1 mol seems sufficient to provoke maximum inhibition. It is difficult to know if this 1 mol of ADP is bound to a single specific site or if it is statistically distributed between

several sites. Besides, it cannot be excluded that the high-affinity ADP binding site (not apparently involved in the inhibition) must be saturated before the binding of ADP to the inhibitory site could occur.

Since ~ 2 mol of ADP can be bound and only ~ 1 is sufficient to inhibit the enzyme, our results are not incompatible with the alternating-site mechanism proposed by Kayalar et al. (1977); F_1 would then contain at least two catalytic and two control sites which is in agreement with the total number of 4–6 mol of *p*-(fluorosulfonyl)benzoyl-adenosine (Esch & Allison, 1978; Di Pietro et al., 1979) or 5 mol of ADP or adenylyl imidodiphosphate (Garret & Penefsky, 1975a) that can bind per mol of F_1 -ATPase.

(2) Is the control ADP binding site one of tight binding? If tightly bound nucleotides are operationally defined as nucleotides not removed by repeated ammonium sulfate precipitations of the enzyme (Harris et al., 1973), the ADP bound during preincubation seems to be linked in a different way. Being not readily reversible in some of the studied conditions, the entrapped ADP may appear tightly bound. In fact, it is not tightly bound since gel filtration in the absence of magnesium is sufficient to remove the bound [14 C]ADP and since [14 C]ADP is not exchanged with tightly bound nucleotides. With nucleotide-depleted F_1 ~ 1 mol of [14 C]ADP is not released by ammonium sulfate precipitation although the full activity is recovered following such treatment. This then cannot be the ADP bound to the control site. Therefore, F_1 bearing the tightly bound nucleotides behaves in the same way as nucleotide-depleted F_1 . Harris et al. (1978) have suggested that the inhibition induced after preincubation with ADP could be due to the replacement of a tightly bound ATP by ADP. This proposal is not in agreement with our results. Indeed, our enzyme preparation is virtually devoid of tightly bound ATP [<0.3 mol/mol (Penin et al., 1979)], and [14 C]ADP is removed from F_1 by ammonium sulfate precipitation.

(3) Is the control ADP binding site the catalytic site? Since the addition of MgATP does not release the [14 C]ADP bound during the preincubation, it cannot be bound to the same site as MgATP. Additional support for this conclusion is brought indirectly by the fact that inhibitors of the ATPase activity such as DCCD (Pougeois et al., 1979) or Nbf-Cl (Ferguson et al., 1975a,b) do not modify the binding of [14 C]ADP. However, it has not yet been proved unambiguously that these inhibitors act directly at the catalytic site.

(4) Is ADP entrapped on the α or the β subunit and how is (are) its site(s) related to regulatory sites? No direct evidence is available to prove the localization of this ADP. However, several results argue in favor of a localization in the vicinity of or on the α subunit. We have seen that Nbf-Cl does not modify the binding of [14 C]ADP; Lunardi & Vignais (1979) have shown that Nbf-Cl prevents the labeling of arylazido-ADP on the β subunit; if arylazido-ADP and unmodified ADP behave similarly, it is likely that this ADP is entrapped at the level of the α subunit. This conclusion is also in agreement with the model proposed by Slater et al. (1979) in which "the catalytic site for ATP hydrolysis is present on the β subunit and a regulatory anion-binding site is on the α subunit. According to this model, ADP in the absence of Mg^{2+} would bind equally well to the α and β subunits; in the presence of Mg^{2+} , ADP would bind preferentially to the regulatory site" as suggested by the binding of 8-azido-ADP (Wagenvoort et al., 1979). If their interpretation is correct, the control site could then be the "regulatory site".

The existence of regulatory site(s) has indeed been proposed previously on the basis of kinetic experiments (Godinot et al.,

1975; Ebel & Lardy, 1975; Schuster et al., 1975; Leimgruber & Senior, 1976; Recktenwald & Hess, 1977; Di Pietro et al., 1979). The binding of ADP to the control site could very well account at least partly for the regulations observed in kinetic experiments.

Harris et al. (1978) have shown by using various ADP analogues that the inhibitions induced during preincubation are very specific for ADP. The same specificity was found for the regulatory sites by kinetic (Schuster et al., 1975) or binding (Pedersen, 1975) studies. This again argues for the identity between control and regulatory sites.

It should be mentioned that the entrapment of ADP only takes place when the quaternary structure of the enzyme is maintained, since no binding occurs after dissociation of the enzyme by cold denaturation (Penefsky & Warner, 1965). Thus, a binding on the free α or β subunit is presumably not possible under the present conditions used to dissociate the enzyme.

(5) Is magnesium alone responsible for the transition between the highly active and the partially inactive form of the enzyme (F_1^*)? Mg^{2+} or other divalent cations provoke the entrapment of ADP in the control sites(s). It is known that Mg^{2+} alone induces a time-dependent inactivation of the membrane-bound or the soluble ATPase (Catterall & Pedersen, 1972; Moyle & Mitchell, 1975; Hackney, 1979; Minkov et al., 1979). This inhibition seems to be very different from that induced by ADP since ADP alone apparently provokes the same kind of inhibition as $MgADP$ but to a lesser extent. One could evoke the role of the tightly bound Mg^{2+} (Senior, 1979); however, this is inconsistent with the fact that ADP binding and concomitant inhibition are easily reversed by gel filtration in the absence of Mg^{2+} .

Recently, Lowe et al. (1979) reported that preincubation with Mg^{2+} followed by ATP addition inhibited ATPase activity independently of the ADP produced; the contradiction between their results and ours may come from their very different conditions and from the enzyme preparation (Beechey et al., 1975) which yields a low specific ATPase activity possibly due to the presence of the natural ATPase inhibitor. The enzyme used in this work, prepared according to Penin et al. (1979) or Di Pietro et al. (1975) with high ATPase activity, contains no proteic inhibitor as demonstrated by the method of Brooks & Senior (1971).

Further work is needed to determine the possible role of the natural ATPase inhibitor in the binding of ADP to the control site. The binding of phosphate, described by Penefsky (1977), is also magnesium-dependent and exhibits similarities to ADP binding. As an attractive working hypothesis, one can imagine that ATP synthesis occurs when ADP and phosphate are present together at this (these) site(s). It is proposed that either the control and the synthetic sites are identical or the binding of ADP (and phosphate?) on this (these) site(s) is responsible for the activation of ATP synthesis.

Acknowledgments

Thanks are due to Bernadette Duclot for typing the manuscript and to Eric Frey for the drawings.

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