Interaction between Catalytic and Regulatory Sites of Mitochondrial F_1 Adenosine-5′-triphosphatase As Monitored by the Differential Effects of Inhibitors and Nucleotide Analogues on the "Hysteretic" Behavior of the Enzyme†

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ABSTRACT: 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSBA) has been shown to bind both at catalytic and regulatory binding sites of F_1-ATPase from pig heart mitochondria [Di Pietro, A., Godinot, C., Martin, J. C., & Gautheron, D. C. (1979) *Biochemistry 18*, 1738–1745]. A stable covalent binding of about 4 mol of [^{14}C]FSBA/mol of F_1 accounts for almost complete inactivation. Preincubation of F_1-ATPase with 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (FSBG) prevents the binding of more than 1 mol of [^{14}C]FSBA/mol of enzyme. Since FSBG is expected to bind only at catalytic sites, this would suggest the existence of at least two catalytic sites. Preincubation of F_1-ATPase with adenosine 5′-diphosphate (ADP) has been shown to induce a "hysteretic" inhibition. This inhibition is characterized by biphasic kinetics of adenosine 5′-triphosphate (ATP) hydrolysis and is due to the slowly reversible binding of ADP at regulatory sites [Di Pietro, A., Penin, F., Godinot, C., & Gautheron, D. C. (1980) *Biochemistry 19*, 5671–5678]. Modification of F_1-ATPase by *N*,*N*′-dicyclohexylcarbodiimide (DCCD) before ADP addition modifies neither the ADP-induced hysteretic inhibition nor the concomitant binding of ADP, whatever the extent of inactivation by DCCD. On the contrary, modification by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine efficiently lowers the hysteretic inhibition and the concomitant binding of ADP. The prevention of hysteretic inhibition depends on the extent of FSBA-induced enzyme inactivation; it becomes complete for high degrees of inactivation. Simultaneously, the kinetics of ATP hydrolysis progressively lose their biphasic pattern and become linear. Under these conditions the binding of [^{14}C]-ADP is decreased from 2.4 to 1.3 mol/mol of F_1 or from 3.3 to 2.0 mol/mol of nucleotide-depleted F_1. If added after ADP, FSBA reverses the ADP binding from 2.4 to 1.4 mol/mol of F_1 or 3.3 to 2.2 mol/mol of nucleotide-depleted F_1; the concomitant hysteretic inhibition loses its biphasic kinetics of ATP hydrolysis. A partial protective effect of ADP against FSBA-induced inactivation is observed. FSBG does not appreciably modify the ADP binding at the regulatory sites whereas it prevents, as efficiently as FSBA, the hysteretic inhibition. This would indicate that (i) prevention of ADP binding by FSBA is due to direct binding at the regulatory sites and not to a conformational change induced by binding at the catalytic sites and (ii) covalent binding at the catalytic sites is sufficient to prevent the hysteretic inhibition induced by ADP, which shows the interdependence of these two types of sites.

It is now widely recognized that mitochondrial F_1-ATPase contains both catalytic and regulatory nucleotide binding sites. The presence of regulatory sites has been recently shown by kinetic studies (Godinot et al., 1975; Ebel & Lardy, 1975; Schuster et al., 1975; Recktenwald & Hess, 1977; Roveri et al., 1980; Fleury et al., 1980) or by measuring the direct binding of ADP (Hilborn & Hammes, 1973; Pedersen, 1975) or nucleotide analogues (Garrett & Penefsky, 1975a,b; Di Pietro et al., 1979; Wagenvoord et al., 1979). Moyle & Mitchell (1975) have suggested as an hypothesis that the binding of ADP to a regulatory site might be involved in transitions from a catalytically active state to an inactive one. However, no experimental data allowed discrimination between catalytic and regulatory sites.

Irreversible inhibition of ATPase activity has been achieved by using chemical modifiers such as *N*,*N*′-dicyclohexylcarbodiimide (DCCD)[1] (Pougeois et al., 1979) or 4-chloro-7-nitrobenzofurazan (Nbf-Cl) (Ferguson et al., 1975) or by using covalent adenine nucleotide analogues. The analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) previously used to covalently label the catalytic sites (Esch & Allison, 1978a,b) has been shown to also bind at regulatory sites (Di Pietro et al., 1979). Very recent results from our laboratory (Di Pietro et al., 1980) have demonstrated "hysteretic" behavior of F_1-ATPase to be the following: The slow binding of ADP induces a progressive inhibition of ATP hydrolysis; this inhibition and the ADP binding can be slowly reversed by ATP in the absence of Mg^{2+}. The binding of ADP occurs at regulatory sites but hydrolysis of ATP at catalytic sites is necessary to observe this inhibition. According to the studies of Schuster et al. (1975), Pedersen (1975), and Harris et al. (1978), the regulatory sites are very selective for adenine nucleotides while the catalytic site(s) is (are) not specific for the heterocyclic base moiety. We therefore used 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (FSBG), an analogue of FSBA in which adenosine is replaced by guanosine, to differentiate the catalytic from the regulatory sites.

The present paper will study the effects of inhibitors of the ATPase activity on the hysteretic behavior of the enzyme.

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[1] Abbreviations used: ATPase, adenosine-5′-triphosphatase; F_1, pig heart mitochondrial F_1-ATPase prepared according to the procedure of Penin et al. (1979), omitting the last step (gel filtration in the presence of 50% glycerol); nucleotide-depleted F_1, pig heart mitochondrial F_1-ATPase prepared according to Penin et al. (1979); BF_1, bacterial soluble F_1-ATPase; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; FSBG, 5′-[*p*-(fluorosulfonyl)benzoyl]-guanosine; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Mops, 3-(*N*-morpholino)propanesulfonic acid; Me_2SO, dimethyl sulfoxide.

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DCCD does not modify the hysteretic inhibition or the concomitant binding of ADP in any way. On the contrary, FSBA efficiently prevents the ADP-induced inhibition and related binding or reverses them when added after ADP. FSBA directly binds both at the ADP inhibitory sites and at the catalytic sites. FSBG does not modify the ADP binding but prevents the hysteretic inhibition induced by ADP. The irreversible binding of FSBG at the catalytic sites prevents ATP binding and its hydrolysis and thus is sufficient to abolish the hysteretic inhibition induced by ADP. These results will be discussed in view of the relationships between catalytic and regulatory sites.

Experimental Procedures

Materials. Nucleotides were purchased from Boehringer Mannheim, [U-^{14}C]ADP (510 mCi/mmol) came from the Radiochemical Centre, Amersham, England. Their purity was monitored either by high-pressure liquid chromatography or by thin-layer ascending chromatography in 0.7 M LiCl as previously described (Di Pietro et al., 1979, 1980). *N,N'*-Dicyclohexylcarbodiimide (DCCD) was obtained from Aldrich. Unlabeled or [*benzoyl*-^{14}C]FSBA (0.46 mCi/mmol) was prepared as previously described (Di Pietro et al., 1979).

Stability of FSBA in Aqueous Buffers. The release of the fluoride ion due to reaction of FSBA (solubilized in dimethyl sulfoxide) with various buffers was measured by using a fluoride-specific electrode (Tacussel). The standard curves were conducted in each buffer with aliquots of 4 or 40 mM potassium fluoride from 10^{-5} to 10^{-3} M. The reaction always followed first-order kinetics. In 40 mM Tris–H_2SO_4–1 mM EDTA, pH 7.5, containing 15% dimethyl sulfoxide, the half-life of 1 mM FSBA was 5.3 h at 30 °C. Addition of 22% glycerol or 0.25 M sucrose largely decreased it to 1.4 and 1.25 h, respectively. Increasing pH also descreased the half-life, which became 2.5 times lower at pH 8.0. Long-term dissociation of the sulfonyl–fluoride bond was accompanied by precipitation of FSBA. ADP or ATP (1 mM) did not significantly modify the FSBA dissociation whereas 0.2 M phosphate largely enhanced it.

Synthesis of FSBG. The method of Pal et al. (1978) was used, starting from guanosine hydrochloride and *p*-(fluorosulfonyl)benzoyl chloride. Guanosine hydrochloride was obtained from guanosine (Boehringer) in an 87% yield. The compound *p*-(fluorosulfonyl)benzoyl chloride (Aldrich) was reacylated as described by Esch & Allison (1978a) in step 3 of FSBA synthesis. Esterification in freshly distilled hexamethylphosphoric triamide led to FSBG with a 16% yield. Its purity was checked by thin-layer chromatography on silica gel F-254 with a solvent system composed of methyl ethyl ketone–acetone–water (65:20:15). A single spot, R_f 0.64, was obtained while guanosine and guanosine hydrochloride migrated with an R_f of 0.30. The ultraviolet absorption spectrum of ethanolic FSBG solution showed two shoulders at 275 and 252 nm, with a $\epsilon_{275}/\epsilon_{252}$ ratio of 0.74. These results agree with those of Pal et al. (1978). The NMR spectrum measured in deuterated dimethyl sulfoxide showed the characteristic peaks: δ 4.2–4.8 [m, 5 protons, $H_{2'}$, $H_{3'}$, $H_{4'}$, $H_{5'}$ (2)], 4.8–5.3 [m, 2.5, $OH_{2'}$ (1), $OH_{3'}$ (1), OH_6 (0.5)], 5.7–5.8 [d, 1, $H_{1'}$ (anomer)], 6.4–6.8 (s, 2, NH_2), 8.1 (s, 1, H_8), 8.2 (s, 4, aromatic), and 10.8–11.0 (s, 0.5, NH_1). The presence of about 2 mol of H_2O (δ 3.9) and about 0.1 mol of hexamethylphosphoric triamide (δ 2.5) per mol of FSBG was also detected; this was taken into account for molecular weight calculations. The melting point, about 190 °C, was thus lower than that reported by Pal et al. (1978). The stability of 1 mM FSBG was determined in our experimental conditions, i.e., 40 mM Tris–H_2SO_4, 1 mM EDTA, and 5% dimethylsulfoxide, pH 7.5, by measuring the release of fluoride ion as for FSBA. A half-life of 110 min was found at 30 °C.

Enzyme Preparation. Pig heart mitochondria were obtained at 0–4 °C as previously described (Gautheron et al., 1964). The mitochondrial F_1-ATPase was purified by the method of Penin et al. (1979), omitting (F_1) or including (nucleotide-depleted F_1) gel filtration on Ultrogel ACA 34 in the presence of 50% glycerol which removed tightly bound nucleotides. F_1 contained 1.8 ± 0.2 mol of ADP and 0.3 ± 0.03 mol of ATP/mol and had a specific activity of 85–95 units/mg of protein (1 unit = 1 μmol of ATP hydrolyzed per min); it was stored in 40 mM Tris–H_2SO_4, 1 mM EDTA, and 2 mM ATP, pH 7.5, as an ammonium sulfate suspension at 0–4 °C. The nucleotide-depleted F_1 was virtually devoid of tightly bound nucleotides (0.17 ± 0.18 mol of ADP and 0.27 ± 0.04 mol of ATP/mol). It exhibited a specific activity of 110–120 units/mg of protein and was stored frozen at −80 °C in 100 mM Tris–H_2SO_4, 5 mM EDTA, and 50% glycerol, pH 8.0.

Just before use, an aliquot of F_1 was centrifuged at 9000*g* for 5 min at room temperature. The pellet was dissolved in 40 or 100 mM Tris–H_2SO_4, and 1 mM EDTA, pH 8.0 (5–10 mg of protein/mL). Saturated ammonium sulfate (1.5 volumes) containing 5 mM EDTA was added, and the sample was centrifuged again. The step was repeated 3 more times and the final pellet was dissolved in 40 or 100 mM Tris–H_2SO_4, 1 mM EDTA, and 10% glycerol, pH 7.5. The enzymatic solution was desalted by the elution–centrifugation method described by Penefsky (1977). When the nucleotide-depleted F_1 was used, the stock solution was diluted 5 times in 100 mM Tris–H_2SO_4, pH 7.5, so that the final EDTA and glycerol concentrations were respectively 1 mM and 10%.

The protein content of enzyme solutions was estimated by the procedure of Lowry et al. (1951) with bovine serum albumin as the standard. The molecular weigth of F_1-ATPase was taken as 380 000 (Di Pietro et al., 1975).

Assay of ATPase Activity. The measurements were performed on 0.5–10-μL aliquots at 30 °C in 0.62 mL of 50 mM Tris–maleate buffer, pH 8.0, 3.3 mM ATP, and 3.3 mM $MgSO_4$, using an ATP regenerating system (phosphoenolpyruvate, pyruvate kinase, lactic dehydrogenase, and NADH) and following spectrophotometrically the rate of NADH disappearance at 340 nm (Pullman et al., 1960).

Preincubation of the Enzyme with ADP for "Hysteretic" Inhibition and Binding Measurements. For studies on the inhibition of ATPase activity induced by [^{14}C]ADP and the concomitant ADP-binding, the enzyme (1–2 mg of protein/mL) was generally preincubated at room temperature in 40 mM Tris–H_2SO_4 and 10% glycerol, pH 7.5, in the presence of 0.2 mM [U-^{14}C]ADP (20 mCi/mmol) and 1.5 mM $MgSO_4$ for 20 min. An aliquot (0.5–10 μL) was transferred into the spectrophotometer cuvette containing the medium of ATPase assay (0.62 mL final volume), and the rate of NADH disappearance was recorded for several minutes.

The binding of [U-^{14}C]ADP was studied by the elution–centrifugation method described by Penefsky (1977). An aliquot (50–100 μL) was layered on the top of a column of Sephadex G-50 fine equilibrated in 40 mM Tris–H_2SO_4–1.5 mM $MgSO_4$, pH 7.5. The radioactivity of the filtrate was measured in 10 mL of a scintillation cocktail composed of 100 g of naphthalene, 5 g of 2,5-diphenyloxazole, 200 mL of water, and 1 L of dioxane.

Inactivation of F_1-ATPase before Preincubation with ADP. F_1-ATPase (2–4 mg of protein/mL) was inactivated at 30 °C either by DCCD (2 mM in methanol) or by FSBA or FSBG

(2 mM in dimethyl sulfoxide), respectively, in 50 mM Tris–50 mM Mops–2 mM EDTA–10% glycerol, pH 7.0, or 40 mM Tris–H_2SO_4–1 mM EDTA, pH 7.5. The residual ATPase activity was measured at intervals. When the inhibition reached the indicated value, 100-μL aliquots were freed from the unbound inhibitor by the elution–centrifugation method in 50 mM Tris–50 mM Mops–1.5 mM $MgSO_4$–10% glycerol, pH 7.0, in the case of DCCD or 40 mM Tris–H_2SO_4–1.5 mM $MgSO_4$ ± 10% glycerol, pH 7.5 in the case of FSBA or FSBG. Controls were conducted by replacing DCCD with 4% methanol and FSBA or FSBG with 5% dimethyl sulfoxide. The eluates were supplemented with $MgSO_4$ (1.5 mM final concentration) and [^{14}C]ADP (0.2 mM final concentration) to study the hysteretic inhibition and the concomitant binding as described above.

Reversal of ADP Binding by FSBA. The enzyme (2–4 mg of protein/mL) was preincubated in the presence of 0.5 mM [^{14}C]ADP, with or without 1.5 mM $MgSO_4$, in 40 mM Tris–H_2SO_4–1 mM EDTA–10% glycerol pH 7.5 for 20 min. FSBA (2 mM) was added, and the residual ATPase activity was measured at intervals. The residual binding of [^{14}C]ADP was measured on 100-μL aliquots of the eluate obtained by the elution–centrifugation method, in the presence of 1.5 mM $MgSO_4$, as described above. Controls were made at the same intervals of incubation by replacing FSBA with 4% dimethyl sulfoxide.

Covalent Binding of FSBA. F_1 (1 mg of protein) was incubated at 30 °C in 1 mL of 40 mM Tris–H_2SO_4–1 mM EDTA, pH 7.5, in the presence of 0.5 mM [*benzoyl*-^{14}C]FSBA (0.46 mCi/mmol). At intervals, the residual ATPase activity was measured on 1–5-μL aliquots. The binding of [^{14}C]FSBA was determined on 100 μL. The samples were precipitated by 500 μL of 10% trichloroacetic acid and immediately centrifuged at 9000*g* for 5 min. The pellets were dissolved in 200 μL of 1 N NaOH, precipitated again by 1 mL of 10% trichloroacetic acid, and centrifuged. This step was repeated once, and the final pellets were dissolved in 200 μL of 1 N NaOH. The protein content was measured on 40 μL while 140 μL was used for radioactivity; the latter was measured only after the complete disappearance of chemiluminescence by repeated countings. In these conditions, only very stable covalent binding of FSBA was recovered.

When the effects of preincubation with FSBG were studied on FSBA binding, F_1 (2 mg of protein/mL) was incubated at 30 °C in 100 μL of 40 mM Tris–H_2SO_4–1 mM EDTA, pH 7.5, with 2 mM FSBG. After 200 min, the enzyme lost 99% of the initial activity while the control, conducted in the presence of 5% dimethyl sulfoxide instead of FSBG, was virtually stable. Unbound FSBG was separated from the enzyme by the elution–centrifugation method in the same buffer. The eluates were supplemented with [^{14}C]FSBA (0.5 mM final concentration) and incubated at 30 °C for 20 h, so that the control (where dimethyl sulfoxide replaced FSBG) lost about 95% of its initial activity. The binding of FSBA was measured after precipitation by trichloroacetic acid and dissolution in NaOH as described above.

When the nucleotide-depleted F_1 was used, the buffer was supplemented with 10% glycerol. Since the latter slowed down FSBA binding and inactivition, two more additions of [^{14}C]FSBA were necessary to reach 95% inactivation.

Results

Competition of FSBG and FSBA for the Catalytic Sites. For studies on the stable covalent binding of FSBA, the enzyme, after incubation with the analogues, was precipitated by trichloroacetic acid and solubilized in 1 N NaOH (see

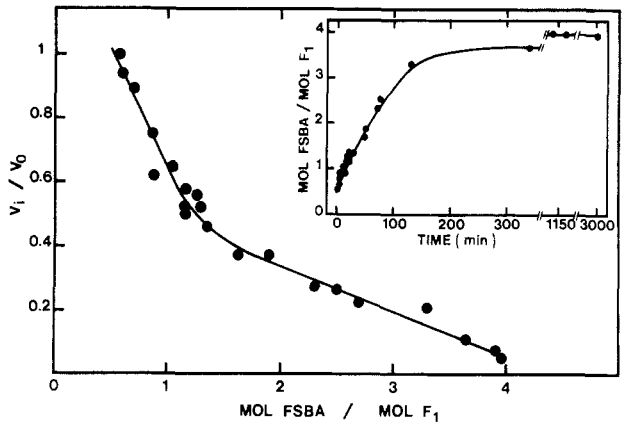

FIGURE 1: Covalent binding of FSBA to F_1 and inactivation. F_1 was incubated with 0.5 mM [^{14}C]FSBA in 40 mM Tris–H_2SO_4–1 mM EDTA, pH 7.5, at 30 °C. At intervals, aliquots were removed, and the residual ATPase activity was measured. The corresponding binding of FSBA was determined after trichloroacetic acid precipitation and solubilization by NaOH as described under Experimental Procedures. v_0 = ATPase activity in the absence of FSBA; v_i = residual ATPase activity after incubation with FSBA. (Insert) Covalent binding of FSBA as a function of incubation time. The zero time value was obtained by introducing [^{14}C]FSBA into the control assay just after the trichloroacetic acid addition.

Experimental Procedures). In these conditions, kinetic studies showed that a maximum of about 4 mol of FSBA/mol of F_1 could be bound (insert, Figure 1), which led to almost complete inactivation instead of the 6 mol of FSBA/mol of F_1 observed under milder conditions (Di Pietro et al., 1979). A biphasic relationship was obtained between this stable covalent binding of FSBA and the inactivation extent of F_1 (Figure 1). Some binding was apparently not involved in the inhibition since about 0.5 mol of FSBA/mol of F_1 was found in the controls when [^{14}C]FSBA was added after trichloroacetic acid addition (zero time); this might correspond to unspecific binding.

Incubation of the enzyme with FSBG resulted in a marked inactivation which was at an even faster rate than that induced by FSBA. The half-time of inactivation by 0.5 mM FSBG was 4.5 min with F_1 or 14 min with nucleotide-depleted F_1 under conditions when the values obtained with the same concentration of FSBA were respectively 13 and 24 min. The FSBG-induced inhibition was reversed neither by a 1000-fold dilution nor by repeated ammonium sulfate precipitations followed by dilution. Gel filtration in the presence of 60 mM K_2SO_4 which removed the tightly bound nucleotides according to Leimgruber & Senior (1976) did not either reverse the inactivation. The activity of the control in which 5% Me_2SO_4 replaced FSBG was retained all along these treatments. Therefore FSBG is neither readily reversible nor tightly bound; it must be covalent.

Preincubation of F_1 with FSBG, leading to a 99% inactivation, prevented the further stable covalent binding of about 1.7 mol of FSBA/mol since the binding was lowered from about 4 to 2.3 mol/mol (Table I). When nucelotide-depleted F_1 was 95% inactivated by FSBG, the binding of FSBA was lowered from about 4.7 to 3.4 mol/mol. These results would indicate that both types of the enzyme contain at least two catalytic sites where both FSBA and FSBG are able to bind.

Prevention by FSBA and FSBG of the ADP-Induced Hysteretic Inhibition. Figure 2 shows the comparative effects of FSBA, FSBG, and DCCD on the ADP-induced hysteretic inhibition. As reported recently (Di Pietro et al., 1980), preincubation of F_1-ATPase with ADP led to the hysteretic inhibition of the ATPase activity (Figure 2, panel A). Contrarily to the control which hydrolyzed ATP at a constant rate,

Table I: Effects of Preincubation with FSBG on the Covalent Binding of [^{14}C]FSBA [a]

enzyme preparation	mol of [^{14}C]FSBA/mol of enzyme: control [b]	mol of [^{14}C]FSBA/mol of enzyme: FSBG-modified enzyme
F_1	3.98 ± 0.18 (4) [c]	2.34 ± 0.19 (4) [c]
nucleotide-depleted F_1	4.69 ± 0.23 (3)	3.45 ± 0.16 (3)

[a] The incubation with 2 mM FSBG was conducted either in 40 mM Tris–H_2SO_4–1 mM EDTA, pH 7.5, for F_1 or in the same buffer plus 10% glycerol for nucleotide-depleted F_1. After 200 min, the extent of inactivation was 99% for F_1 or 95% for nucleotide-depleted F_1. Unbound FSBG was eliminated by the elution–centrifugation method, and [^{14}C]FSBA was added. The binding of FSBA was measured after 20 h, the time of incubation corresponding to a 95% FSBA-induced inactivation of the controls (see Experimental Procedures). [b] The controls were carried out in parallel experiments where 5% dimethyl sulfoxide replaced FSBG. [c] Results are presented as the mean followed by the standard error of the mean; the number of experiments is in parentheses.

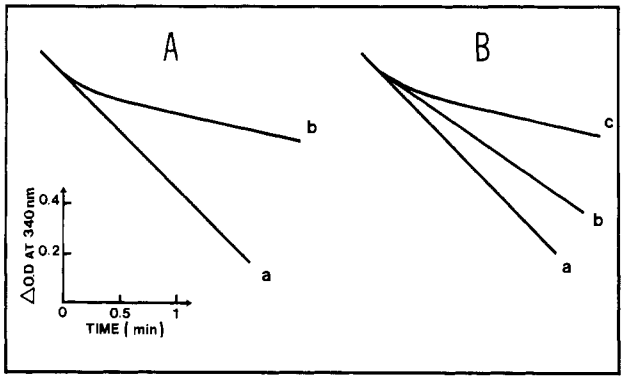

FIGURE 2: Effects of inactivation of F_1 by inhibitors of ATPase activity on the ADP-induced hysteretic inhibition. (Panel A) Typical recordings of the rate of ATP hydrolysis after preincubation of F_1 with or without ADP. F_1 (1 mg of protein/mL) was preincubated in 40 mM Tris–H_2SO_4, 1.5 mM $MgSO_4$, pH 7.5, and ±10% glycerol at 30 °C in the absence (a) or presence (b) of 200 μM ADP. After a 20-min preincubation, 0.5-μL aliquots (0.5 μg of protein) were added to the standard ATPase assay medium, and the rate of ATP hydrolysis was followed spectrophotometrically at 340 nm (see Experimental Procedures). (Panel B) Before preincubation with ADP, F_1 (2 mg of protein/mL) was first inactivated to 5% of its original activity by incubation either with 2 mM DCCD for 240 min in 50 mM Tris–50 mM Mops–2 mM EDTA–10% glycerol, pH 7.0, or with 2 mM FSBA or 2 mM FSBG for 90 min in 40 mM Tris–H_2SO_4–1 mM EDTA, pH 7.5. The free inhibitor was eliminated by the elution–centrifugation procedure (see Experimental Procedure) in the same buffers, and the modified enzyme was preincubated with 1.5 mM $MgSO_4$ in the absence (a) or in the presence (b, c) of 200 μM ADP. The residual activity was measured on 10-μL aliquots (10 μg of protein). (a) Controls, no ADP; (b) FSBA-modified or FSBG-modified F_1 + ADP; (c) DCCD-modified F_1 + ADP.

the sample first preincubated with ADP and Mg^{2+} showed biphasic kinetics. During the first few seconds, the ATP hydrolysis started at a rate identical with that of the control but progressively diminished until a steady-state inhibited rate was reached, always within 1 min after the initiation of the reaction. The ADP-induced hysteretic inhibition was never complete, the maximal inhibition being respectively 76% for F_1 and 85% for nucleotide-depleted F_1.

Previous inactivation of F_1 either by DCCD, FSBA or FSBG to a 95% extent (Figure 2, panel B) led to the following observations: (i) The inhibitors alone did not produce biphasic kinetics of ATP hydrolysis (trace a); i.e., they did not themselves, as ADP did, induce hysteretic inhibition. (ii) ADP addition to the DCCD-modified enzyme normally developed hysteretic inhibition since the biphasic kinetics and the same

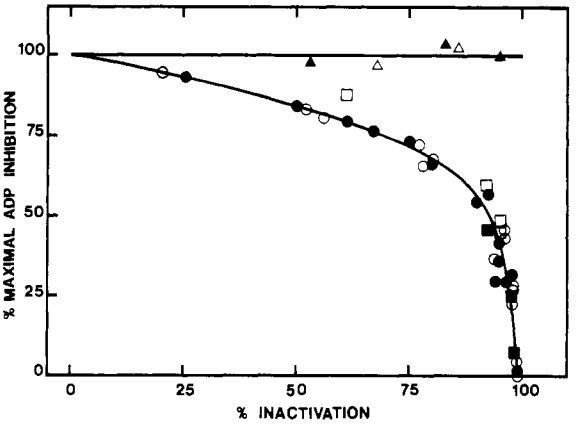

FIGURE 3: Prevention of the ADP-induced hysteretic inhibition as a function of the extent of inactivation by inhibitors. The conditions were the same as in Figure 1 (panel B) except that the incubation of the enzyme with 2 mM DCCD (▲, △), FSBA (●, ○), or FSBG (■, □) was stopped at various times. The corresponding residual activities were measured as in Figure 1B (trace a); the percentage of inactivation was calculated by comparison with the value obtained in Figure 1A (trace a). $MgSO_4$ (1.5 mM) and ADP (200 μM) were incubated for 20 min with the modified enzyme, and the steady-state inhibited rate was measured as in Figure 1B, (trace b or c). The percentage of maximal ADP-induced inhibition was calculated by the ratio slope of trace (b or c × 100)/(slope of trace a), all traces being obtained as in Figure 1B. Experiments were conducted either with F_1 (▲, ●, ■) or with nucleotide-depleted F_1 (△, ○, □). The maximal ADP-induced inhibition was 76% for F_1 and 85% for nucleotide-depleted F_1 (percentages calculated by comparison of traces a and b in Figure 1A).

extent of inhibition (78% with respect to the control) were observed (trace c). (iii) With the FSBA-modified enzyme, the ADP-induced inhibition was much lower. The kinetics were almost linear, and the inhibition was reduced to 32% (trace b). (iv) The prevention by FSBG of the ADP-induced inhibition was approximately as efficient as that obtained with FSBA. Results very similar to those reported for F_1 in Figure 2 were obtained with nucleotide-depleted F_1.

The residual activity after DCCD inactivation of the enzyme exhibited the same percentage of ADP-induced hysteretic inhibition, whatever the extent of inactiviation (Figure 3). On the contrary, the prevention of the ADP-induced inhibition by FSBA was dependent on the extent of modification, that is, on the degree of enzyme inactivation by FSBA (Figure 3). The higher the inactivation by FSBA was, the lower the ADP-induced inhibition. The abolition of the ADP-induced inhibition became complete for a very high inactivation degree. Although the maximal ADP-induced inhibition was higher for nucleotide-depleted F_1 than for F_1, the same pattern was obtained for both types of this enzyme. As illustrated in Figure 2, the kinetics of ATP hydrolysis lost their biphasic character, and quite linear kinetics were obtained when the ADP-induced inhibition was lowered to less than 5% of its original value. A high degree of inactivation by FSBA was necessary to significantly decrease the ADP-induced inhibition since the inhibition was only 16% lower when the enzyme was 50% FSBA inactivated. Half the maximal prevention was obtained after 93% FSBA inactivation. A very similar prevention of ADP-induced inhibition was obtained when FSBG replaced FSBA.

Influence of DCCD, FSBA, and FSBG on the ADP Binding at Regulatory Sites. As reported previously (Di Pietro et al., 1980), the ADP remaining bound to F_1-ATPase after gel filtration is responsible for the hysteretic inhibition; the inhibition was suggested to be due to the binding of ADP at regulatory sites. The effects of FSBA which binds to both

Table II: Effects of Inactivation by DCCD, FSBA, or FSBG on the Binding of ADP to Regulatory Sites Responsible for Hysteretic Inhibition [a]

inhibitor	F_1 inactivation (%)	F_1 mol of [^{14}C]ADP/mol [b]	nucleotide-depleted F_1 inactivation (%)	nucleotide-depleted F_1 mol of [^{14}C]ADP/mol [b]
none		2.4 ± 0.17 (9) [c]		3.29 ± 0.13 (11) [c]
DCCD	50	2.4	50	3.3
	95	2.4	90	3.3
FSBA	50	2.0	52	2.8
	95	1.6	95	2.2
	99	1.3	99	2.0
FSBG			64	3.2
	93.5	2.05	93	3.2
	99	2.0	96.5	3.15

[a] Inactivation by 2 mM DCCD, FSBA, or FSBG was conducted under the same conditions as those used in Figure 2. [b] The enzyme was incubated with 0.2 mM [^{14}C]ADP and 1.5 mM $MgSO_4$. The amount of ADP remaining bound after gel filtration was measured after elimination of the free ADP by the elution–centrifugation method as described under Experimental Procedures. [c] The number of experiments is indicated in parentheses. In the other cases, results are the mean of at least two separate experiments.

Table III: Reversion of ADP Binding and Concomitant Hysteretic Inhibition by Incubation with FSBA [a]

enzyme preparation	incubation time (min)	mol of [^{14}C]ADP/mol of enzyme control	mol of [^{14}C]ADP/mol of enzyme +FSBA	inactivation by FSBA (%)
F_1	0	2.22 ± 0.30 (4)		0
	10		2.06	12 (45) [b]
	30		1.90	49 (80)
	200	2.11	1.40	78 (95)
nucleotide-depleted F_1	0	3.19 ± 0.31 (4)		0
	15		2.84	14 (50) [b]
	50		2.56	23 (80)
	230	3.01	2.20	59 (95)

[a] The enzyme was preincubated with 0.5 mM [^{14}C]ADP in the absence of Mg^{2+}, in 40 mM Tris–H_2SO_4–1 mM EDTA–10% glycerol, pH 7.5, for 20 min, and the steady-state inhibited rate of ATPase activity was measured. FSBA (2 mM) was added (or replaced by 4% dimethyl sulfoxide in the control), and the residual activity was measured at the indicated times. The binding of [^{14}C]ADP was determined after separation of the free [^{14}C]ADP by the elution–centrifugation method in the presence of 1.5 mM $MgSO_4$ and in the absence of ADP. [b] The percentages of inactivation induced by FSBA in parallel experiments conducted in the absence of ADP are in parentheses. The ATPase activities at zero time were respectively 36 or 81 units/mg of proteins for F_1 and 26 or 105 units/mg of protein for nucleotide-depleted F_1 in the presence or absence of ADP.

catalytic and regulatory sites were investigated on the binding of ADP to these regulatory sites.

In controls, the binding of [^{14}C]ADP was found to be 2.4 mol of ADP/mol of F_1 and 3.3 mol of ADP/mol of nucleotide-depleted F_1 (Table II). Inactivation of either F_1 or nucleotide-depleted F_1 by DCCD did not modify the binding of [^{14}C]ADP, whatever the extent of the inactivation. On the contrary, the binding of ADP was diminished in the FSBA-modified enzyme. For a high degree of FSBA inactivation, corresponding to almost complete prevention of the hysteretic inhibition, F_1 could only bind 1.3 mol of ADP/mol instead of 2.4 mol of ADP/mol in the control while nucleotide-depleted F_1 could bind 2.0 mol of ADP/mol instead of 3.3 mol of ADP/mol. When [^{14}C]ADP and Mg^{2+} were added to the FSBG-modified enzyme, only a slight decrease of ADP binding was observed (Table II) as compared with that induced by FSBA, even for very high degrees of inactivation.

The effects of FSBA or FSBG on the ADP-induced hysteretic inhibition were dependent on the nucleoside moiety of the molecule. Indeed incubation of the enzyme with *p*-(fluorosulfonyl)benzoyl chloride, which did not itself appreciably inactivate F_1-ATPase compared to FSBA or FSBG at the same concentration, neither modified the ADP binding nor the concomitant hysteretic inhibition. The results were similar with both kinds of the enzyme.

Reversal by FSBA of the ADP-Induced Hysteretic Inhibition and Concomitant Binding of ADP. Since Mg^{2+} appeared essential to ensure ADP entrapment into the regulatory sites (Di Pietro et al., 1980), the preincubation in the reversal experiments was conducted in the absence of added Mg^{2+}. However, Mg^{2+} was present in the elution–centrifugation technique to permit the measurement of ADP binding.

Preincubation of F_1-ATPase with [^{14}C]ADP and further incubation with FSBA resulted in a lowered ADP binding, depending on the time of incubation with FSBA (Table III). The binding was diminished from 2.2 to 1.4 mol of ADP/mol of F_1 and from 3.2 to 2.2 mol of ADP/mol of nucleotide-depleted F_1 for a 3–4-h incubation with FSBA (dissolved in Me_2SO) while the binding in the control, where Me_2SO alone replaced FSBA, was scarcely modified. As the reversal of ADP binding increased, the kinetics of ATP hydrolysis progressively lost their biphasic character. The inhibition of ATPase activity induced by FSBA in the presence of ADP was much lower than that observed in the absence of ADP, indicating that ADP afforded appreciable protection of the enzyme against FSBA inactivation. In fact, this protective effect is complex since it reflects a competition between an irreversible inactivator (FSBA) and a reversible inhibitor (ADP) to occupy a site, the former progressively chasing the latter our of the site.

If Mg^{2+} was present both in the preincubation medium and in the elution–centrifugation columns, FSBA had no significant effect on [^{14}C]ADP binding during the course of the experiments. Indeed FSBA no longer appeared accessible to sites where [^{14}C]ADP was entrapped in the presence of Mg^{2+}.

Discussion

The adenine nucleotide analogue FSBA, which reacts covalently with F_1-ATPase, efficiently prevents ADP binding at the regulatory sites involved in the hysteretic behavior of the enzyme or reverses the binding when added after ADP. This prevention or reversion is due to direct binding of FSBA at the ADP regulatory sites.

Esch & Allison (1978b) have concluded that under their conditions, FSBA binds specifically at the catalytic sites on β subunits. These authors assumed that the curvature of the semilog plot of F_1-ATPase inactivation kinetics was due to artifacts since (1) higher pHs decreased the linear phase of the plot, (2) a second addition of FSBA enhanced the inhibition, and (3) 3′-FSBA had a very short half-life (37 min). In fact, (1) the effects of pH are difficult to interpret since pH also affects enzyme ionization. (2) A second addition of inhibitor resulted in a much lower burst of ATPase inhibition, and (3) as shown by Colman et al. (1977), 5′-FSBA is much more stable than 3′-FSBA. Furthermore, we used a fluoride-specific electrode under our precise conditions, that is, 40 mM Tris–H_2SO_4–1 mM EDTA, pH 7.5, in the absence of glycerol or sucrose to avoid the slowing down of FSBA inactivation (see Experimental Procedures). A half-life of 5.3 h for 5′-FSBA, which led to minor corrections of FSBA concentration during the course of our experiments, was found.

The FSBA inactivation kinetics obtained under these conditions (Di Pietro et al., 1979) are thus effectively biphasic and reflect the binding of FSBA at both catalytic and regulatory sites. Such biphasic kinetics of FSBA inactivation have also been reported for rabbit muscle pyruvate kinase (Wyatt & Colman, 1977) or bacterial carbamoyl phosphate synthetase (Boettcher & Meister, 1980).

The total amount of FSBA bound per mole of enzyme depends on the method used. We previously reported (Di Pietro et al., 1979) that about 6 "irreversible" mol of FSBA/mol of F_1 was recovered after gel filtration or ethanol precipitation, that is, under relatively mild conditions. We show here that only about 4 "stable covalent" mol of FSBA is found after acidic precipitations followed by alkaline solubilizations. Such a value was also found by Esch & Allison (1978b) under similar denaturating conditions. The difference of 2 mol of FSBA/mol of F_1 is therefore due either to noncovalent but firm binding or to covalent binding, less stable in acidic or alkaline conditions (Gold, 1967). In any case, a biphasic curve of inactivation of F_1 as a function of the amount of FSBA bound is found, indicating that two kinds of sites are involved. This biphasic curve might reflect the binding of FSBA at both catalytic and regulatory nucleotide binding sites since Esch & Allison (1978b) proved that FSBA bound at catalytic sites and we showed (Di Pietro et al., 1979) that is also binds at regulatory sites. FSBG must also be covalently bound since the FSBG-induced inhibition could never be reversed. Besides, pretreatment of the enzyme with FSBG prevented the stable covalent binding of more than 1 mol of FSBA. This result was not unexpected since FSBG should exhibit the same chemical reactivity as FSBA, which has been widely used as a covalent affinity label. FSBG was also covalently bound in the case of glutamate dehydrogenase (Pal et al., 1978).

The inefficiency of FSBG in ADP binding, as opposed to the efficiency of FSBA, indicates the direct binding of the latter at the regulatory sites which are selective for the adenine nucleotides (Schuster et al., 1975; Pedersen, 1975; Harris et al., 1978). The covalent binding of FSBG to the catalytic sites, less selective (Ebel & Lardy, 1975; Schuster et al., 1975), however, seems sufficient to prevent the ADP-induced hysteretic inhibition. It should be kept in mind that to observe the ADP-induced hysteretic inhibition, the substrate must first be hydrolyzed at the catalytic site (Di Pietro et al., 1980). The behavior of FSBG shows the mutual dependence of catalytic and regulatory sites and confirms the model recently proposed (Di Pietro et al., 1980) in which the hysteretic inhibition requires the hydrolysis of Mg-ATP.

The lack of effect of Nbf-Cl or DCCD on the ADP binding (Di Pietro et al., 1980) indicates that they do not bind at the nucleotide regulatory sites. Although they have been reported to completely inactivate F_1-ATPase (Ferguson et al., 1975; Pougeois et al., 1979), which might suggest binding at catalytic sites, their effects on nucleotide or analogue binding are not clear. Indeed, the binding of about 0.8 mol of DCCD/mol of BF_1 scarcely lowers the [^{14}C]ADP binding but abolishes the ATP-induced quenching of aurovertin fluorescence (Satre et al., 1979). However, the binding of 1.6 mol of DCCD/mol of F_1 prevents both the ADP-induced enhancement and the ATP induced quenching of aurovertin fluorescence (Pougeois et al., 1979). The binding of about 1 mol of Nbf-Cl/mol of BF_1 has been reported to prevent the ATP-induced quenching of aurovertin fluorescence (Lunardi et al., 1979) whereas this was not the case with F_1 (Ferguson et al., 1976). Nbf-Cl seems to prevent the binding of 1 mol of 3′-O-[4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]-ADP to the β subunit of F_1 (Lunardi & Vignais, 1979) whereas it does not modify the total binding of FSBA (Esch & Allison, 1979). Our results do not exclude DCCD (or Nbf-Cl) binding into the catalytic sites. If they actually do bind, every DCCD-modified molecule of F_1 should have no activity at all. On the contray, F_1 keeps some residual activity when FSBG is the modifier. Under these conditions, the ADP-induced hysteretic inhibition observed in the first case would be due only to the molecules of F_1 not modified by DCCD, whereas in the second case, both FSBG-modified and -unmodified enzyme molecules would participate in the hysteretic inhibition. The enzyme could be partly modified by FSBA or FSBG since the inactivation of the enzyme is correlated with the binding of several moles of analogue. This partial modification could suppress the hysteretic inhibition without inhibiting completely the ATPase activity.

Additional information supporting the recently proposed model of the hysteretic behavior of F_1-ATPase (Di Pietro et al., 1980) is given by the present study. (1) FSBA prevents the binding of about 1 mol of ADP when the hysteretic inhibition is completely prevented. This proves that about 1 mol of ADP is involved in the hysteretic phenomenon and agrees with previous results. The latter showed that in the presence of dimethyl sulfoxide, the binding of only about 1 mol of ADP to F_1 was sufficient to induce the maximal inhibition. (2) The failure of FSBA to reverse the ADP bound in the presence of Mg^{2+}, as opposed to the easy reversion when Mg^{2+} was absent, suggests that Mg^{2+} entraps ADP in its regulatory binding site; ADP is thus no longer exchangeable with the external medium. This was previously suggested by the easy reversal of ADP binding by gel filtration in the absence of Mg^{2+} (Di Pietro et al., 1980). (3) The apparent contradictory effects of FSBG, which does not appreciably modify the ADP binding but prevents as efficiently as FSBA the ADP hysteretic inhibition, indicate the existence of interactions and mutual dependence between catalytic and regulatory sites. The hysteretic inhibition due to ADP binding at regulatory sites requires hydrolysis of Mg-ATP at the catalytic sites in order to be observed.

Preincubation of the enzyme with FSBG before FSBA modification suggests the existence of at least two catalytic sites. This is compatible with previous results (Wagenvoord et al., 1977; Di Pietro et al., 1979; Esch & Allison, 1979). The binding of FSBG only at these catalytic sites induces a higher extent of inactivation than the binding of FSBA at both catalytic and regulatory sites. This suggests that the FSBA bound at the regulatory sites would protect the enzyme against FSBA binding at the catalytic sites. Thus saturation of both the regulatory sites by ADP and the catalytic sites by the substrate is necessary to have hysteretic inhibition.

Experiments are in progress to determine the significance of these regulations in situ on the synthesis and hydrolysis of ATP.

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Interaction of Calmodulin with Skeletal Muscle Myosin Light Chain Kinase†

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ABSTRACT: Studies on myosin light chain kinase isolated from rabbit skeletal muscle show that the enzyme has a molecular weight of 80 000-84 000 with a sedimentation coefficient of 3.2 S and an apparent Stokes radius of 53 Å. Gel filtration chromatography with a ^{3}H-labeled calmodulin using a Hummel-Dryer technique shows that the enzyme will bind 1 mol of calmodulin per mol of enzyme, with an affinity of $(1.9 \pm 0.5) \times 10^7$ M^{-1} in the absence of substrate. The calmodulin dependence of enzyme activation at limiting Mg^{2+} and light chain concentrations confirms this observation. The calcium dependence of activation of the enzyme-calmodulin complex is characterized by a Hill coefficient of 2.5, with half-activation occurring at 6.6×10^{-7} M Ca^{2+}. The amino acid composition shows a high percentage (9.1%) of proline, which may account for the large apparent Stokes radius and no clear resemblance to other skeletal muscle proteins. A comparison of the amino acid composition with that from turkey gizzard shows some resemblance.

Phosphorylation of the 18 500-dalton light chain component of myosin (LC_2)[1] from rabbit fast skeletal muscle is catalyzed by a Ca^{2+}-dependent myosin light chain kinase (MLCK).[1] In intact skeletal muscle, this occurs during contraction (Bárány et al., 1979) and may play a role in the posttetanic potentiation of peak twitch tension in the muscle (Manning & Stull, 1979).

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[1] Abbreviations used: LC_1, light chain 1; LC_2, light chain 2; LC_3, light chain 3; MLCK, myosin light chain kinase; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Mops, 4-morpholinopropanesulfonic acid; Me_2SO, dimethyl sulfoxide; Caps, 3-(cyclohexylamino)propanesulfonate; DTE, dithioerythritol; Hap (HAP in figures), hydroxylapatite; Tris, tris(hydroxymethyl)aminomethane.

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