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Role of phosphate on the ADP-induced hysteretic inhibition of mitochondrial adenosine 5′-triphosphatase. Effects of the natural protein inhibitor

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Preincubation of F_1-ATPase with ADP and Mg^{2+} leads to ADP binding at regulatory site inducing a hysteretic inhibition of ATP hydrolysis, i.e., an inhibition that slowly develops after Mg-ATP addition (Di Pietro, A., Penin, F., Godinot, C. and Gautheron, D.C. (1980) Biochemistry 19, 5671–5678). It is shown here that inorganic phosphate (P_i) together with ADP during preincubation abolishes the time-dependence of the inhibition after the addition of the substrate Mg-ATP. This preincubation in the presence of both P_i and ADP slowly leads to a conformation of the enzyme immediately inhibited after the addition of the substrate Mg-ATP. The P_i effect is half-maximal at 35 μM and pH 6.6, whereas a limited effect is induced at pH 8.0. The preincubation of F_1-ATPase with P_i and ADP must last long enough ($t_{1/2} = 5$ min). The effects can be correlated to the amount of P_i bound to the enzyme, 1 mol P_i per mol (apparent K_D of 33 μM) at saturation. P_i neither modifies the ADP binding nor the final level of the concomitant inhibition. When P_i is not present in the preincubation, the final stable rate of ADP-induced hysteretic inhibition is always reached when a near-constant amount of P_i has been generated during Mg-ATP hydrolysis. Kinetic experiments indicate that preincubation with ADP and P_i decreases both V_{max} and K_m which would favor a conformational change of the enzyme. Taking into account the P_i effects, a more precise model of hysteretic inhibition is proposed. The natural protein inhibitor IF_1 efficiently prevents the binding of P_i produced by ATP hydrolysis indicating that the hysteretic inhibition and the IF_1-dependent inhibition obey different mechanisms.

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Abbreviations: ATPase, adenosine-5′-triphosphatase, E.C. 3.6.1.3; F_1-ATPase, soluble mitochondrial adenosine-5′-triphosphatase; nucleotide-depleted F_1, pig heart mitochondrial F_1-ATPase prepared according to Penin et al. (Ref. 20); IF_1, natural protein inhibitor of mitochondrial ATPase; AMP-P(NH)P, adenosine-β,γ-imidotriphosphate; GMP-P(NH)P, guanosine-β,γ-imidotriphosphate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethylpropane)-1,3-diol; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Introduction

It has been well established that F_1-ATPase from mitochondria, bacteria or chloroplasts possesses multiple binding sites for nucleotides and P_i (see the recent reviews, Refs. 1–4). It was previously shown that binding of ADP to a regulatory site [5], specific for adenine nucleotides [6–8], induces a hysteretic inhibition, i.e., a progressive inhibition which leads to a stable low-rate after about 1 min of Mg-ATP hydrolysis. This ADP site has been recently localized on a β subunit [9].

Several types of P_i binding sites have been described. A high-affinity site was suggested as playing a catalytic role in ATP synthesis [10] and could be equivalent to the γ-phosphate position of an ATP site [11]. A high-affinity site has been localized on the β subunit with a photoactivable analogue of P_i [12,13]. Additional, low affinity P_i binding sites have been reported [14]. It has been shown in previous work that low-affinity P_i sites might regulate the overall conformation of the enzyme inducing differential reactivity to chemical modification [15] and to trypsin proteolysis [16].

Diverse effects of P_i on the binding of ADP, depending on the conditions used and the source of F_1-ATPase, have been reported. P_i was found to decrease the binding of ADP to the bacterial enzyme [17]. In the case of the chloroplastic enzyme, P_i also was observed to decrease ADP binding [18] whereas no effect was obtained with a photoactivable analogue of P_i [13]. In the case of the mitochondrial enzyme, no effect at all was induced whether P_i [10] or its photoactivable analogue [12] was used.

The aim of the present work was to investigate the role of P_i in the ADP-induced hysteretic inhibition of pig heart mitochondrial F_1-ATPase activity and the concomitant ADP binding. It is shown that P_i does not modify the ADP binding related to hysteretic inhibition. On the contrary, P_i is necessary, in addition to ADP, to induce the final inhibition of ATPase activity. Indeed, when P_i is present during preincubation with ADP, a linear inhibited-rate is immediately observed upon addition of the substrate. The effect of P_i corresponds to a saturation binding of one mol P_i per mol enzyme at a high-affinity site. In contrast when P_i is absent from the preincubation medium, the setting up of the hysteretic inhibition is dependent on the P_i generated by Mg-ATP hydrolysis and thus a lag phase is observed. The inhibition induced by both ADP and P_i leads to a conformation of the enzyme with a lower rate of ATPase activity and a lower K_m for Mg-ATP. The natural protein inhibitor IF_1 efficiently prevents the P_i binding generated by ATP hydrolysis.

Materials and Methods

Materials. Nucleotides were purchased from Boehringer Mannheim. [U-^{14}C]ADP, 510 mCi/mmol, came from the Radiochemical Center, Amersham, U.K. Their purity was checked by thin-layer ascending chromatography on poly(ethyleneimine) cellulose with 0.7 M LiCl. [^{32}P]P_i, 10 mCi/ml (or 10 mCi/250 μl) and carrier-free were from the Commissariat à l'Energie Atomique, France. The Gamma Prep-A kit used to prepare [γ-^{32}P]ATP from [^{32}P]P_i came from Genofit S.A., Genève, Switzerland. Pyruvate kinase and lactate dehydrogenase as 50% glycerol solutions were from Boehringer Mannheim. Ultrogel AcA 54 was from IBF.

Biological preparations. Pig heart mitochondria were obtained as previously described [19]. The nucleotide-depleted F_1 was purified by the method of Penin et al. [20]. It was stored at −70°C in 100 mM Tris-H_2SO_4 (pH 8.0) containing 5 mM EDTA and 50% glycerol. Just before use, an aliquot was thawed, diluted in the appropriate buffer at pH 8.0 or 6.6 and equilibrated in the same buffer by centrifugation-elution through a Sephadex G-50 column [10]. The protein inhibitor IF_1 was purified from pig heart mitochondria [21] by the method of Ref. 22. Analysis by SDS polyacrylamide gel electrophoresis indicated an apparent relative molecular mass identical to that of bovine IF_1 kindly provided by Prof. Y. Hatefi, La Jolla, California, U.S.A.

The protein content of solutions was estimated by the method of Lowry et al. [23] as modified [24] using bovine serum albumin as a standard. The relative molecular weight for pig heart mitochondrial F_1-ATPase was taken as 380 000 [25].

Assay of ATPase activity. Spectrophotometric measurement were performed as previously described [8] in either 50 mM Hepes (KOH) (pH

8.0) or 50 mM Mes (Tris) and 25 mM KCl (pH 6.6). When kinetics of ATPase activity were studied, equal concentrations of ATP and $MgCl_2$ from 0.05 to 3.4 mM were used. In all cases, a 1 mM excess of $MgCl_2$ was added for full activity of the auxiliary enzyme system and Mg-ATP concentrations were calculated assuming a stability constant of 167000 [26].

[^{32}P]P_i binding. Nucleotide-depleted F_1 (7.7 mg/ml) was diluted 10-fold and equilibrated in 0.25 M sucrose/10 mM Mops (Tris)/10% glycerol/1 mM $MgSO_4$ (pH 6.6). The enzyme was incubated in the presence of 50 μM ADP and increasing [^{32}P]P_i concentrations, from 5 to 100 μM, containing a constant amount of radioactivity of $3 \cdot 10^6$ cpm. After 30 min at 30°C, each assay (100 μl) was applied to the top of a Sephadex G-50 (fine) column and eluted by centrifugation [10,14]. The eluates were analyzed for their protein and radioactivity content. The latter was estimated with a Beckman LS 7000 liquid scintillation counter: 50-μl eluates were counted in 5 ml water. Controls were performed under the same conditions except that the enzyme was omitted.

Binding of ^{32}P species as generated from [γ-^{32}P]ATP hydrolysis. [γ-^{32}P]ATP was synthesized enzymatically [27] from [^{32}P]P_i at 10 mCi/250 μl. It was mixed with 10 mM cold ATP to a specific radioactivity of $0.2–0.4 \cdot 10^6$ cpm/nmol. The enzyme (77 μg/100 μl) in 0.25 M sucrose/10 mM Mops (Tris)/10% glycerol/1 mM $MgSO_4$ (pH 6.6) was preincubated either with 0.2 mM [γ-^{32}P]ATP for 30 min (equivalent to 0.2 mM $^{32}P_i$ and ADP) or with 50 μM ADP for 30 min and further incubated with 0.2 mM [γ-^{32}P]ATP for 2 min (standard conditions for the setting up of hysteretic inhibition). In both cases, the total amount of bound [^{32}P] species was estimated by centrifugation-elution. The eluates were collected in tubes containing 24 μl 10% (w/v) sodium dodecylsulfate. Cold P_i and ATP (5 μmol) were added as carriers and [^{32}P]P_i and [γ-^{32}P]ATP were estimated after isobutanol-benzene extraction [28] as modified [29].

Measurement of [^{14}C]ADP binding concomitant to the hysteretic inhibition. The previously published method was used [5,8] in the presence or absence of P_i.

Chemical modification by DCCD. Nucleotide-depleted F_1 (1.0 mg/ml) in 0.25 M sucrose/10 mM Mops (Tris)/10% glycerol/2 mM EDTA (pH 6.6) was incubated for 90 min at 30°C in the presence of 2 mM DCCD (solution in methanol, final concentration 2% in assay). Unbound DCCD was eliminated by centrifugation-elution through a column equilibrated in 0.25 M sucrose/10 mM Mops (Tris)/10% glycerol/1 mM $MgSO_4$ (pH 6.6). A control assay was performed in the presence of methanol but in the absence of DCCD. The residual ATPase activity of the unmodified or the DCCD-modified enzyme was measured by the spectrophotometric method, as described above.

Results

Effects of P_i at pH 6.6 on the kinetics of ADP-induced hysteretic inhibition

When equilibrated at 0.6–0.9 mg/ml in a buffer at either pH 8.0 or 6.6, the hydrolytic activity of the nucleotide-depleted F_1 (initially kept at pH 8.0) was not significantly different. This activity was, however, very dependent on the pH of the ATPase assay medium and the presence of 10 mM (sodium) bicarbonate as an activating anion. A rate of 56–62 μmol ATP hydrolyzed/min per mg was obtained in 50 mM Hepes (KOH) (pH 8.0) which was enhanced to a value of 81–91 by addition of 10 mM (sodium) bicarbonate (Fig. 1, lower recording). A much lower rate of 19–23 μmol ATP hydrolyzed/min per mg was measured in 40 mM Mes (Tris) (pH 6.6) (Fig. 1B, lower recording) and the activity showed a very low sensitivity to the activating anion, since a limited activation of about 10% was observed. This might be attributed to the low amount of HCO_3^- species at pH 6.6 ($pK_{a_1} = 6.4$). An intermediate activity, with an intermediate sensitivity to the activating anion, was obtained at pH 7.0 (not shown). Preincubation of the enzyme for 30 min in the presence of Mg^{2+} and 400 μM P_i did not modify the ATPase activity whether measured at pH 8.0 or 6.6 (Fig. 1, lower recordings). When 50 μM ADP replaced P_i during preincubation, biphasic kinetics of Mg-ATP hydrolysis, characteristic of the hysteretic inhibition [5], were observed (Fig. 1, middle curves). During the first few seconds, the Mg-ATP hydrolysis started at a rate similar to that of the control and then progressively diminished to reach

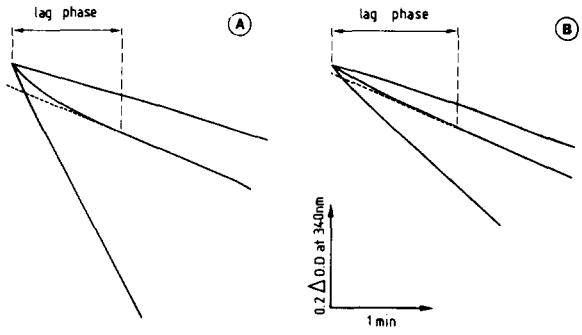


Fig. 1. Recordings of Mg-ATP hydrolysis by nucleotide-depleted F_1 after preincubation at pH 6.6 with ADP and P_i. The enzyme (0.6 mg/ml) in 0.25 M sucrose, 10 mM Mops (Tris), 10% glycerol, 1 mM $MgSO_4$ (pH 6.6) was preincubated with or without 400 μM P_i (lower curves), which 50 μM ADP alone (middle curves) or with both ADP and P_i (higher curves). After 30 min, the ATPase activity was measured in a final volume of 0.62 ml in the presence of 10 mM $NaHCO_3$ at either pH 8.0 on 0.6-μl aliquots (panel A) or at pH 6.6 on 2-μl aliquots (panel B), as described in Materials and Methods. The dotted lines were drawn from the steady-state rates to allow graphical extrapolation of the half-lag time of hysteretic inhibition (see the text).

a stable inhibition rate. When the difference in activity between the experimental value and that of the dotted line extrapolated from the final steady-state rate was studied in semi-log plots as a function of reaction time, a straight line was obtained. This indicated that the lag phase before the steady-state rate was exponential. Half-lag time values of 0.18 and 0.28 min were graphically determined for ATPase activity, respectively, at pH 8.0 and 6.6, under conditions of Fig. 1, middle recordings.

When both ADP and P_i were present during preincubation (Fig. 1, upper traces), immediate linear kinetics of inhibited Mg-ATP hydrolysis were obtained indicating that the presence of P_i during preincubation suppressed the lag phase of the ADP-induced hysteretic inhibition. It should be noticed that the immediate linear rate observed in the presence of ADP and the rather high concentration of 400 μM P_i was even lower than the final inhibited rate induced by ADP alone, whereas it was about equal at 100 μM P_i. When P_i was added in the ATPase assay medium (1–2 min) instead of the preincubation medium (30 min), no significant effect was produced (not shown). As a control, the presence of a P_i trap, composed of glyceraldehyde 3-phosphate, NAD^+, glyceraldehyde-3-phosphate dehydrogenase, pyruvate and lactate dehydrogenase, added before P_i, prevented the effect of P_i against the lag phase of hysteretic inhibition. The same P_i effects toward the ADP-induced hysteretic inhibition were observed in the absence of glycerol with the native enzyme (containing tightly bound nucleotides).

Dependence of P_i effects on P_i concentration and on the pH of preincubation medium

Fig. 2 clearly shows that the concentration dependence of P_i effects greatly differed whether the pH of preincubation was 6.6 or 8.0. At pH 6.6 (closed symbols) when P_i is essentially under the monoionic form, the half-lag time of the setting up of ADP-induced hysteretic inhibition rapidly

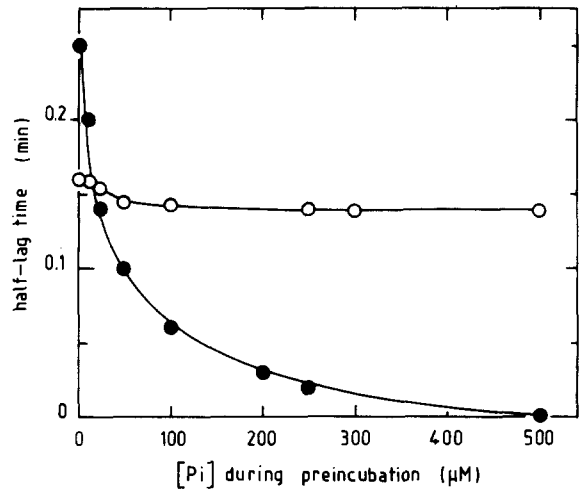


Fig. 2. Effects of pH and P_i concentration during preincubation with ADP on the half-lag time further required to reach the stable inhibited-rate of hydrolysis after Mg-ATP addition to the ATPase assay. Nucleotide-depleted F_1 (0.56 mg/ml) was equilibrated either in 50 mM Hepes (KOH)/10% glycerol/1 mM $MgSO_4$ (pH 8.0) (○) or in 0.25 M sucrose/10 mM Mops (Tris)/10% glycerol/1 mM $MgSO_4$ (pH 6.6) (●). After preincubation for 30 min at 30 °C with 50 μM ADP and increasing amounts of P_i, the residual ATPase activity was measured at the corresponding pH on 1 μl (pH 8.0) or 2 μl (pH 6.6). The half-lag time necessary to reach the stable inhibited-rate of ATP hydrolysis was determined as indicated in Fig. 1, middle curves, and plotted as a function of P_i concentration during preincubation.

decreased when P_i concentration increased during preincubation. The recordings of Mg-ATP hydrolysis progressively lost their concavity to become completely linear. The half-maximal effect was obtained with 35 μM P_i whereas 250 μM P_i produced about 90% of the maximal effect. In contrast, only limited lowering in the half-lag time of inhibition was produced at pH 8.0 (open symbols), when P_i is essentially under the diionic form, even at high concentrations that also yield significant amounts of the monoionic form. The same limited lowering in half-lag time at pH 8.0 was obtained when the Hepes (KOH) buffer (Fig. 2) was replaced by Tris (Mes) or Tris (HCl). When the experiment was performed at pH 7.0 (data not shown), intermediate results were observed, since a 40% or a 68% decrease in the half-lag time was produced by 35 or 250 μM P_i, respectively.

Dependence of P_i effects on the preincubation time

The effects of P_i during preincubation are not immediate, since, as mentioned above, no significant effect on the ADP-induced hysteretic inhibition was observed when P_i was added in the ATPase assay medium. It is shown in Fig. 3 (closed symbols) that the time of preincubation of the enzyme in the presence of P_i (and ADP) must be long enough to suppress the lag phase of hysteretic inhibition after addition of the substrate. A full effect was obtained after 30 min, while half-maximal effect was reached at about 5 min. As a control, the enzyme first preincubated with ADP and further incubated in the absence of P_i (Fig. 3, open symbols) retained the same half-lag time of hysteretic inhibition all along the time interval of preincubation studied.

Binding of P_i related to abolition of the lag phase of hysteretic inhibition

When the nucleotide-depleted F_1 was preincubated for 30 min at pH 6.6 in the presence of 1 mM $MgSO_4$, 50 μM ADP and increasing [^{32}P]P_i concentrations up to 100 μM, a saturation curve of binding was obtained and linear double reciprocal plots (Fig. 4) allowed to extrapolate a maximal binding of 0.95 mol P_i/mol enzyme with an apparent K_D of 33 μM. The amount of bound P_i was linearly correlated to the decrease of the half-lag time of ADP-induced hysteretic inhibition

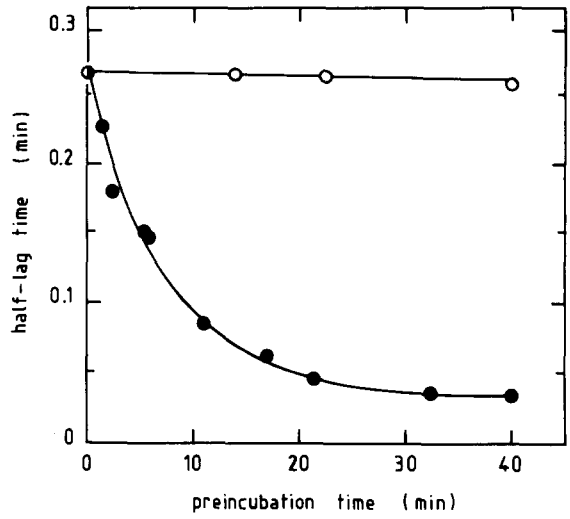


Fig. 3. Effect of the time of preincubation with P_i on the half-lag time of ADP-induced hysteretic inhibition. Nucleotide-depleted F_1 (0.6 mg/ml) was equilibrated in 0.25 M sucrose/10 mM Mops (Tris)/10% glycerol/1 mM $MgSO_4$ (pH 6.6) and preincubated with 50 μM ADP at 30°C. After 30 min, the solution was mixed (●) or not (○) with 200 μM P_i. The ATPase activity was measured at pH 6.6 in the presence of 10 mM $NaHCO_3$ and was followed as a function of the preincubation time with P_i. The half-lag time of the recordings was estimated as in Fig. 1, middle curves.

(insert Fig. 4): a binding of 0.86 mol [^{32}P]P_i was obtained during preincubation with 100 μM P_i which produced a 75% decrease of half-lag time, while a complete abolition could be reached for an extrapolated value of 1.1 mol P_i bound/mol enzyme. When the enzyme was first loaded with 0.86 mol [^{32}P]P_i bound/mol (maximal amount experimentally bound in the presence of 100 μM [^{32}P]P_i as shown in Fig. 4) and then incubated for 1 min with 3 mM cold ATP just before the centrifugation elution, a value of 0.74 mol [^{32}P]P_i bound/mol was recovered indicating that the bound P_i was not markedly chased by about 1500 turnover of the enzyme. On the contrary, the P_i binding was largely prevented by addition to the preincubation medium of 50 μM AMP-P(NH)P, a non hydrolysable analogue of ATP (not shown here).

Since ATP, which was readily hydrolyzed during the 30 min-preincubation in the presence of Mg^{2+}, was able to produce the same hysteretic inhibition as added ADP + P_i, the binding of [^{32}P]P_i as generated by [γ-^{32}P]ATP hydrolysis was

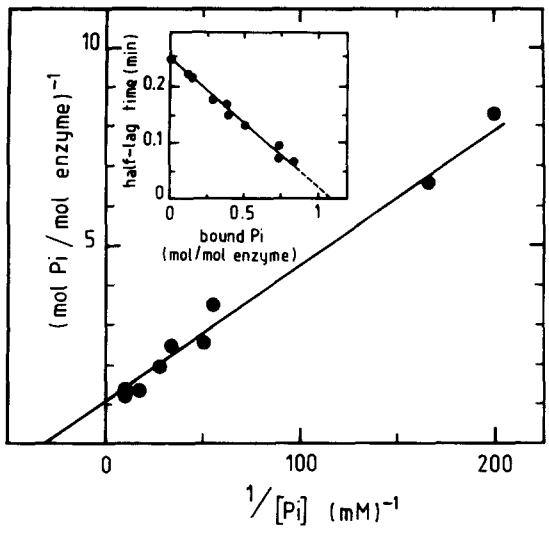


Fig. 4. [^{32}P]P_i binding at pH 6.6 in the presence of ADP. Nucleotide-depleted F_1 (0.77 mg/ml) was incubated at pH 6.6 in the presence of 5–100 μM [^{32}P]P_i and 50 μM ADP for 30 min. The binding of [^{32}P]P_i was measured as described in Materials and Methods. Control experiments indicated that up to 100 μM P_i, no more than 4% of bound [^{32}P]P_i was eluted in the absence of the enzyme under the conditions used. Inset: correlation between [^{32}P]P_i binding and the decrease of the half-lag time of ADP-induced hysteretic inhibition. At each concentration of added [^{32}P]P_i, the binding was correlated to the effect on the half-lag time estimated as in Fig. 2.

also investigated. Table I, upper line, shows that when the enzyme was preincubated at pH 6.6 in the presence of 1 mM $MgSO_4$ and 200–500 μM [γ-^{32}P]ATP during 30 min, a total amount of 0.94 mol [^{32}P] species was bound per mol enzyme after centrifugation-elution. They were essentially constituted of [^{32}P]P_i (0.86 mol/mol) and only of trace amount of [γ-^{32}P] ATP (0.08 mol/mol).

Finally, when the enzyme was first preincubated with 1 mM $MgSO_4$ and 50 μM ADP for 30 min and then further incubated with 0.2 mM [γ-^{32}P]ATP for only 2 min, i.e., under conditions leading to the final low-rate of activity due to hysteretic inhibition, only a low amount of [^{32}P]P_i (0.17–0.32 mol/mol) was bound after centrifugation elution. However, higher amounts of [γ-^{32}P]ATP were bound (0.71–0.58 mol/mol) and therefore a total binding of about 0.9 mol ^{32}P species/mol was obtained (not shown here).

TABLE I

BINDING OF [^{32}P]P_i AS GENERATED FROM [γ-^{32}P]ATP HYDROLYSIS - PREVENTION BY IF_1

The nucleotide-depleted F_1 (40 μg) was preincubated in 50 μl 0.25 M sucrose/10 mM Mops (Tris)/10% glycerol/1 mM $MgSO_4$ (pH 6.6) in the absence or the presence of 10 μg IF_1 and 0.5 mM [γ-^{32}P]ATP (216 cpm/pmol). After 30 min at 30°C, the samples were diluted with an equal volume of the same buffer and submitted to centrifugation-elution. The eluates were collected in 2% sodium dodecyl sulfate (final concentration). The total ^{32}P bound was estimated by counting in water. [γ-^{32}P]ATP and [^{32}P]P_i were separated by isobutanol-benzene extraction (see Materials and Methods) and counted. Each value is the mean of three separate experiments.

Effector	Bound label (mol/mol enzyme)		
	Total [^{32}P]	[γ-^{32}P]ATP	[^{32}P]P_i
Mg[γ-^{32}P]ATP	0.94	0.08	0.86
IF_1 + Mg[γ-^{32}P]ATP	0.41	0.30	0.11

Lack of P_i effect on the concentration-dependent ADP-binding concomitant to the hysteretic inhibition

As previously described, preincubation of nucleotide-depleted F_1 with [^{14}C]ADP induced a maximal binding of about 3 mol ADP per mol enzyme, only one of them being directly correlated to hysteretic inhibition [5,7–9]. Fig. 5 shows that

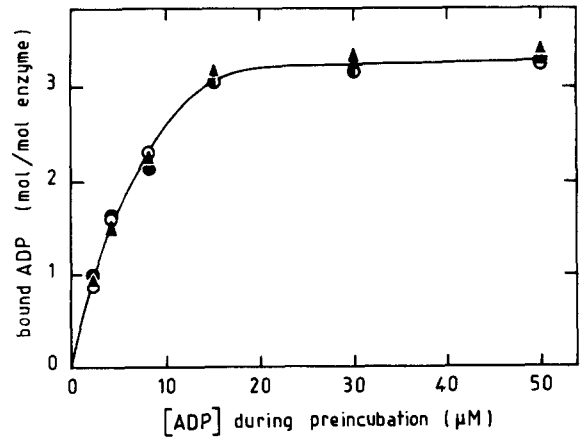


Fig. 5. Binding of [^{14}C]ADP in the presence or absence of P_i. Nucleotide-depleted F_1 (0.85 mg/ml) in 50 mM Mops (Tris)/10% glycerol/1.5 mM $MgSO_4$ (pH 7.0) was incubated for 30 min at 30°C with increasing [^{14}C]ADP concentrations (2–50 μM) in the absence (○) or the presence of 90 μM (●) or 5 mM (▲) P_i. The bound ADP was estimated after centrifugation elution.

addition of 90 μM or 5 mM P_i to the preincubation medium, at any ADP concentration tested, never affected ADP binding although these P_i concentrations decreased the half-lag time of hysteretic inhibition respectively by 52% and 98%, at pH 7.0. The presence of 400 μM P_i during a 30-min preincubation of the enzyme at pH 6.6 did not modify either the setting up of hysteretic inhibition measured as a function of the time of addition of ADP ($t_{1/2}$ = 0.5–1 min).

Modulation of the half-lag time of ADP-induced hysteretic inhibition

Table II indicates that various conditions modifying the initial rate of ATPase activity were able to affect the half-lag time of hysteretic inhibition induced by preincubation with ADP. When the enzyme concentration in the aliquot increased (experiment 1) producing P_i more and more rapidly, the half-lag time decreased. The total amount of ATP hydrolyzed and therefore that of P_i liberated during this period did not markedly vary: it only increased 1.3-fold when the enzyme amount increased 4-fold. In the same way, a pretreatment by DCCD, which partially inactivates the enzyme activity (6.7-fold lowering of the initial activity) but does not modify hysteretic inhibition [5,8], greatly enhanced the half-lag time, but the amounts of P_i liberated by ATP hydrolysis were only 1.5-fold decreased (experiment 2). It should be noticed that the half-lag time was reached at pH 6.6 (experiments 1 and 2) for a P_i concentration of about 6–8 μM produced in the ATPase assay medium. This concentration is much lower than

TABLE II

TIME AND PHOSPHATE DEPENDENCE DURING THE ATPase ASSAY OF THE ADP-INDUCED HYSTERETIC INHIBITION STARTING WITH ENZYME SAMPLES OF VARYING ACTIVITIES.

Nucleotide-depleted F_1 was equilibrated in 0.25 M sucrose/10 mM Mops (Tris)/10% glycerol/1 mM $MgSO_4$ (pH 6.6) and preincubated with 50 μM ADP for 30 min at 30 °C. The ADP-induced hysteretic inhibition was recorded as in Fig. 1, middle curves. The half-lag time necessary to reach the stable inhibited-rate was estimated as in Fig. 1. The amount of P_i generated by ATP hydrolysis during this time was calculated.

Experiment	pH in ATPase assay	Conditions modifying ATPase activity	Half-lag time (min)	P_i generated by ATP hydrolysis during half-lag time	
				(nmol)	concentration (μM)
1	6.6	enzyme amount (μg) [a]			
		0.75	0.30	3.8	6.1
		1.5	0.26	4.3	7.0
		3.0	0.19	4.9	7.9
2	6.6	inactivation by DCCD (%) [b]			
		0	0.27	4.4	7.1
		85	9.0	2.9	4.7
3	8.0	activation by 10 mM HCO_3^- [c]			
		0	0.20	6.0	9.7
		+	0.13	5.8	9.3

[a] In experiment 1, different aliquots (1–4 μl) of the enzyme solution (0.75 mg protein/ml) were used.

[b] Before incubation with ADP, nucleotide-depleted F_1 was first partially inactivated by DCCD in the same medium except that EDTA replaced $MgSO_4$ (see Experimental procedures). Before inactivation, the ATPase activity, at pH 6.6, was 21.2 and 5.7 μmol ATP hydrolyzed/min per mg, respectively, in the absence and the presence of ADP.

[c] Contrary to experiments 1 and 2 where the ATPase activity was measured in Mes buffer (pH 6.6), experiment 3 in the presence of bicarbonate anion was performed in Hepes buffer (pH 8.0), since only a very limited anion activation could be observed at pH 6.6. The activity was measured on 1-μl aliquots (0.75 μg protein). It was 58.8 and 11.5 μmol ATP hydrolyzed/min per mg, respectively, without and with ADP in the absence of anion, and 85 and 12.3 μmol ATP/min per mg in the presence of 10 mM (sodium) bicarbonate. Several experiments of each type 1, 2 or 3 were conducted.

TABLE III

EFFECTS OF PREINCUBATION WITH ADP AND P_i AT pH 6.6 ON KINETIC PARAMETERS OF ATPase ACTIVITY

Nucleotide-depleted F_1 (0.6 mg/ml) was equilibrated by centrifugation elution in 0.25 M sucrose/10 mM Mops (Tris)/10% glycerol/1 mM $MgSO_4$ (pH 6.6) and preincubated for 30 min at 30°C in the presence of 400 μM P_i or 50 μM ADP or both P_i and ADP. The ATPase activity was measured in the presence of increasing concentrations of $MgCl_2$ and ATP (0.05–3.4 mM) either in 40 mM Mes (Tris) (pH 6.6) or in 50 mM Hepes (KOH) (pH 8.0) with 10 mM $NaHCO_3$. Under these conditions no apparent cooperativity was observed and V_{max} and K_m (Mg-ATP) were graphically extrapolated from double-reciprocal plots according to Lineweaver and Burk.

Additions during the 30-min preincubation at pH 6.6	V_{max} (μmol ATP hydrolyzed /min per mg)		K_m(Mg-ATP) (mM)	
	pH 6.6	pH 8.0	pH 6.6	pH 8.0
None	19.2	85.1	0.33	0.24
P_i	19.6	89.6	0.34	0.24
ADP	7.8	21.8	0.15	0.08
ADP + P_i	6.1	16.4	0.12	0.07

that required in the preincubation medium (35 μM in Fig. 2). The experiments of type 3 (Table II) show that when the enzyme activity at pH 8.0 was increased 1.5-fold by 10 mM (sodium) bicarbonate, the half-lag time of hysteretic inhibition was decreased but the amounts of ATP hydrolyzed and P_i produced during this time were not significantly modified.

Change in kinetic parameters of ATPase activity by preincubation at pH 6.6 in the presence of ADP and P_i

After preincubation of nucleotide-depleted F_1 at pH 6.6, measurements of ATPase activity in Mes buffer pH 6.6 gave apparent control values of V_{max} and K_m (Mg-ATP) of 19.2 μmol ATP hydrolyzed/min per mg and 0.33 mM, respectively (Table III). Addition of P_i alone during preincubation did not significantly modify these values. On the contrary, after preincubation with ADP, the stable inhibited-rate reached a maximal value 2.5-fold lower than that of the control and its dependence toward Mg-ATP concentration gave a 2.2-fold

TABLE IV

EFFECTS OF VARIOUS TREATMENTS ON THE REVERSION OF INHIBITION BY ATP ALONE OR IF_1 + ATP

Nucleotide-depleted F_1 (0.42 mg/ml) was incubated in 50 mM Mes (Tris), 10% glycerol, 1 mM $MgSO_4$ (pH 6.6) in the presence of 0.5 mM ATP with or without 55 μg IF_1/ml. After 30 min at 30°C, the residual ATPase activity was measured and 100-μl aliquots were used for each treatment.

Effector in the 30-min preincubation	Activity (μmol ATP/min per mg)					
	before treatment	time of treatment (min)	Centrifugation elution at pH 8.2, EDTA treatment [a]		Ultrogel AcA 54 treatment [b]	
				(% reversion)		(% reversion)
None	59	20	57.5			
		80			61.5	
ATP	19.5	20	62.5	100		
		80	61.5	100	63.5	100
IF_1 + ATP	15.5	20	23	16		
		80	29.5	30	38	47

[a] The samples were submitted to centrifugation-elution at pH 8.2 in 40 mM Tris-H_2SO_4, 10% glycerol, 1.5 mM $MgSO_4$ and then mixed with 5 mM EDTA. The activity was estimated after 20 min or 80 min.

[b] The samples were submitted to a slow Ultrogel AcA 54 gel filtration on a 20×0.6 cm-column equilibrated in 50 mM Mes (Tris)/10% glycerol/1 mM $MgSO_4$ (pH 6.6). The flow-rate was 4 ml/h and the eluates containing the enzyme were obtained after 80 min and used for activity measurements in Hepes buffer (pH 8.0).

lower K_m. When both P_i and ADP were present in preincubation, the effect was even more pronounced with decreases of 3.1-fold in V_{max} and 2.8-fold in K_m. The same kind of results were obtained when the ATPase assay was performed in Hepes buffer (pH 8.0), in the presence of activating anion to suppress negative cooperativity [26,30]. P_i alone did not show any effect, V_{max} and K_m remaining constant to 85–90 μmol ATP hydrolyzed/min per mg and to 0.24 mM, respectively. Decreases of 3.9-fold in V_{max} and 3.0-fold in K_m were observed with ADP, whereas values of 5.2 and 3.4 were obtained with both ADP and P_i, respectively.

Effects of IF_1 on the ADP and P_i-induced hysteretic inhibition

The characteristics of hysteretic inhibition present similarities with the inhibition observed in the presence of IF_1 under specific conditions (see review Ref. 31). Indeed the inhibition with IF_1 requires (i) a rather long preincubation in the presence of Mg^{2+} and ATP, which is hydrolyzed to ADP + P_i, (ii) a slightly acidic pH [22] and (iii) IF_1 binding to a high affinity site on the β-subunit [32]. In addition, IF_1 prevents the release of ADP and ATP bound during preincubation with Mg-ATP [33]. Eventual relationship between the hysteretic inhibition due to IF_1 has therefore been investigated.

It is shown in Table I, lower line, that the addition of IF_1 just before Mg-[γ-^{32}P]ATP in the preincubation drastically lowered the binding of [^{32}P]P_i produced during [γ-^{32}P]ATP hydrolysis. Moreover, when the enzyme preincubated with ADP was mixed with IF_1 before addition of P_i at pH 6.6, higher concentrations of P_i were required to decrease the half-lag time of hysteretic inhibition: a half-maximal decrease was reached at about 250 μM P_i in the presence of IF_1, as compared to 35 μM P_i in its absence, under conditions of Fig. 2 (not shown here).

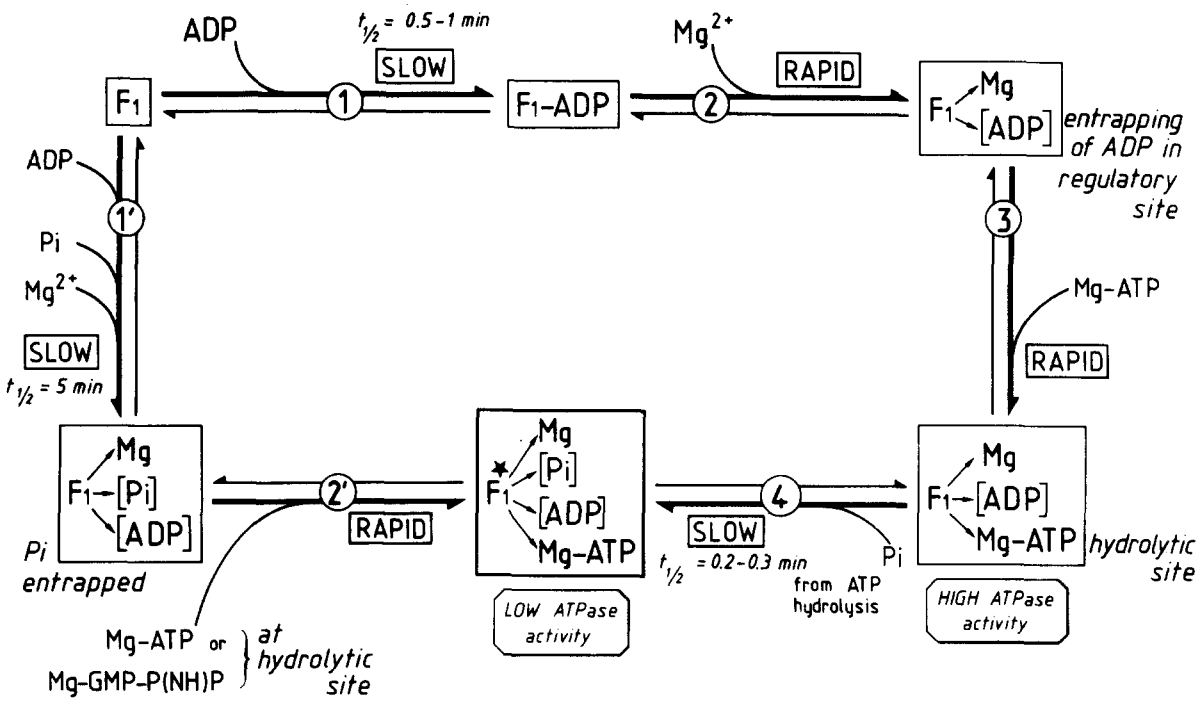
Another important effect of IF_1 was to lower the rate of reversion of the inhibition produced in the presence of Mg^{2+} and ATP. Table IV shows that the hysteretic inhibition due to preincubation with Mg^{2+} and ATP at pH 6.6 was rapidly and completely reversed by increase of pH up to 8.2 during a quick centrifugation-elution. The presence of IF_1 in the preincubation, which produced an additional inhibition, markedly lowered the rate of reversion promoted by the treatment since a only 30% reversion was observed after 80 min. A partial reversion of the inhibition produced by IF_1 + ATP was also reached after a slow gel-filtration (80 min) on Ultrogel AcA 54 at pH 6.6 which completely released the ATP hysteretic inhibition obtained in the absence of IF_1.

Discussion

The present work shows that P_i, in addition to ADP, is required to induce the large inhibition of ATPase activity, previously characterized as hysteretic inhibition [5]. A more precise model including the effects of P_i is proposed to explain the mechanism of hysteretic inhibition of F_1-ATPase activity finally leading to the very compact and inhibited F_1* conformation (Scheme I).

Previous studies have shown that a time-dependent binding of ADP to F_1-ATPase (half-time = 0.5–1 min at pH 6.6) induces a first more compact conformation of the enzyme (step 1) exhibiting a low sensitivity to trypsin proteolysis and to the concomitant inactivation [18]. The binding of one mol ADP only per mol enzyme occurs at a regulatory site specific to adenine nucleotides [6–8] located on a β subunit [9]. The presence of Mg^{2+} prevents ADP to be rapidly released from its site (step 2) and addition of the substrate Mg-ATP (step 3) induces a second slow conformational change occurring during Mg-ATP hydrolysis with a half-time of 0.2–0.3 min (step 4). Considering this slow last step, one can wonder what event triggers the final conformational change leading to the stable inhibited-form F_1*. Is it only the P_i produced by Mg-ATP hydrolysis or the enzyme catalytic turnover or both?

The experimental approach used in the present work has allowed to break away from the enzyme turnover. It is shown here that a preincubation of 30 min with Mg^{2+}, ADP and P_i (step 1′) leads to an enzyme that can be inhibited without lag and that contains at saturation 1 mol [^{32}P]P_i/mol. The binding is not dependent on the enzyme turnover, since the saturation can be reached as well by preincubation with ADP and [^{32}P]P_i, i.e., without turnover, as by hydrolysis of [γ-^{32}P]ATP during



Scheme I. Model of ADP plus P_i-induced inhibition of F_1-ATPase activity. Half-reaction times at pH 6.6.

the preincubation. The inhibited activity of the F_1^* form of the enzyme, characterized by a lower rate of ATPase activity and a lower K_m for Mg-ATP as compared to the initial uninhibited-form of the enzyme, is immediately observed after addition of the substrate Mg-ATP (no lag at step 2'). A lower K_m for Mg-ATP might reflect an increased affinity for the substrate. However, since V_{max} and K_m vary as to keep their ratio about constant, the decrease in K_m could be explained by a Briggs-Haldane mechanism with $k_2 \gg k_{-1}$ as has been shown for the bovine heart enzyme [34,35]. The [^{32}P]P_i bound during preincubation remains bound to the enzyme during the course of Mg-ATP hydrolysis, even after a large number of turnover. It is well known that Mg-GTP is a good substrate for F_1-ATPase and that GMP-P(NH)P, a non hydrolysable analogue, is an efficient competitive inhibitor of both GTP and ATP hydrolysis [36,37]. GMP-P(NH)P has been shown to induce together with ADP and P_i a more compact enzyme conformation; the latter was characterized by a low ATPase activity no longer sensitive to activating anions [38] and by an increased α-helix content of the circular dichroism spectrum [37]. If GMP-P(NH)P really mimics the effects of ATP at the catalytic site, this would mean that the setting up of the final inhibition does not require many turnover of the enzyme, if any, although the catalytic site must be occupied by a nucleoside triphosphate.

It is also shown here that P_i generated during 1–2 min of ATP hydrolysis is responsible for the setting up of the inhibition at step 4, since a near-constant P_i concentration is needed to reach the final F_1^* conformation independently of the enzyme activity.

A number of results, however, show that P_i is much more efficient at step 4 than at step 1' to abolish the lag phase of hysteretic inhibition: (i) the half-maximal concentration is lower (6–8 μM against 35 μM); (ii) the half time is lower (0.2–0.3 min against 5 min); (iii) the amount of bound P_i is lower (0.17–0.32 mol/mol against 0.9–1.0 mol/mol) and (iv) the inhibition is less dependent on the pH (an inhibition is observed at either pH 8.0 or 6.6 at step 4, whereas a slightly acidic pH is mandatory at step 1'). This pH dependence at step

1′ suggests that the monoionic form of P_i (pK_2 in the range 6.9–7.2) is involved. However, since no marked effect is observed with high P_i concentrations at pH 8.0, a role of the (internal) pH itself at step 4 on the accessibility and/or reactivity of the P_i cannot be excluded. A conformational change or some another unknown factor occurring during turnover might also be involved.

As was observed for ADP, the inhibition is related to the binding of only 1 mol P_i per mol enzyme with a high affinity (apparent $K_D = 33$ μM). Also about one mol P_i has been found to bind to the nucleotide-depleted enzyme from bovine heart mitochondria after preincubation in the presence of P_i, Mg^{2+} and low amounts of ADP, although no correlation was made with enzyme activity [39]. Interestingly an opposite stimulatory effect of phosphate at pH 7.5 on ATP hydrolysis under 'uni-site' catalysis conditions was recently reported [40]. However, this interpretation must be taken with caution, since the high phosphate concentration of 50 mM which stimulates more than 10-fold the 'uni-site' hydrolysis is also known to inhibit by about 50% the 'steady-state' hydrolysis [26].

Only a limited amount of the P_i produced by ATP hydrolysis is bound in the presence of IF_1. This indicates that IF_1 likely maintains the P_i site empty by either preventing the P_i binding or producing the immediate release of the transitorily bound P_i. It cannot be excluded, however, that the P_i site might be at least partly occupied by the γ-phosphate moiety of ATP in the presence of IF_1, since triphosphate nucleotides or analogues have been shown to inhibit P_i binding. IF_1 has also been shown to lower at step 1′ the ability of P_i to decrease the lag phase of hysteretic inhibition. This might be correlated to the prevention by IF_1 of the binding of free P_i from the preincubation medium to F_1-ATPase, as shown for the bovine enzyme [10,33].

Another effect of IF_1 is to slow down the rate of reversion of the inhibition produced in the presence of Mg^{2+} and ATP. Therefore IF_1 interferes with hysteretic inhibition but the IF_1-dependent inhibition is of a different type.

In conclusion, it has been clearly established that the binding of 1 mol P_i at a high-affinity site together with the binding of 1 mol ADP per mol enzyme is required to obtain the ADP-induced hysteretic inhibition and to suppress the lag observed in the setting up of the inhibition. The final inhibited-rate is even lower when P_i at high concentration (at least 0.4 mM) is added together with ADP to the preincubation medium (step 1′). This may be due to additional P_i binding at low affinity site(s) [14,16]. It is not known as yet whether these asymmetric P_i and ADP bindings play a catalytic or a regulatory role in the ATP synthesis reaction by the membrane-bound enzyme.

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References

1 Futai, M. and Kanazawa, H. (1983) Microbiol. Rev. 47, 285–312
2 Hammes, G.G. (1983) Trends Biochem. Sci. 8, 131–134
3 Senior, A.E. and Wise, J.G. (1983) J. Membrane Biol. 73, 105–124
4 Vignais, P.V. and Satre, M. (1984) Mol. Cell. Biochem. 60, 33–70
5 Di Pietro, A., Penin, F., Godinot, C. and Gautheron, D.C. (1980) Biochemistry 19, 5671–5678
6 Harris, D.A., Gómez-Fernández, J.C., Klungsoyr, L. and Radda, G.K. (1978) Biochim. Biophys. Acta 504, 364–383
7 Baubichon, H., Godinot, C., Di Pietro, A. and Gautheron, D.C. (1981) Biochem. Biophys. Res. Commun. 100, 1032–1038
8 Di Pietro, A., Godinot, C. and Gautheron, D.C. (1981) Biochemistry 20, 6312–6318
9 Fellous, G., Godinot, C., Baubichon, H., Di Pietro, A. and Gautheron, D.C. (1984) Biochemistry 23, 5294–5299
10 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899
11 Kasahara, M. and Penefsky, H.S. (1977) in Structure and Function of Energy Transducing Membranes (Van Dam, K. and Van Gelder, B.F., eds.), pp. 295–305, Elsevier, Amsterdam
12 Lauqin, G.J.M., Pougeois, R. and Vignais, P.V. (1980) Biochemistry 19, 4620–4626
13 Pougeois, R., Lauquin, G.J.M. and Vignais, P.V. (1983) Biochemistry 22, 1241–1245

14 Kasahara, M. and Penefsky, H.S. (1978) J. Biol. Chem. 253, 4180–4187
15 Di Pietro, A., Godinot, C., Martin, J.C. and Gautheron, D.C. (1979) Biochemistry 18, 1738–1745
16 Di Pietro, A., Godinot, C. and Gautheron, D.C. (1983) Biochemistry 22, 785–792
17 Lunardi, J. (1981) Thesis of Docteur ès Sciences Naturelles, Grenoble, France
18 Dunham, K.R. and Selman, B.R. (1981) J. Biol. Chem. 256, 10044–10049
19 Gautheron, D.C., Durand, R., Pialoux, N. and Gaudemer, Y. (1964) Bull. Soc. Chim. Biol. 46, 645–660
20 Penin, F., Godinot, C. and Gautheron, D.C. (1979) Biochim. Biophys. Acta 548, 63–71
21 Archinard, P., Penin, F., Godinot, C. and Gautheron, D.C., (1986) Methods Enzymol. Biomembranes, part M, Vol 126, Ch. 43, pp. 455–460, Academic Press, New York
22 Horstman, L.L. and Racker, E. (1970) J. Biol. Chem. 245, 1336–1344
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
24 Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250
25 Di Pietro, A., Godinot, C., Bouillant, M.L. and Gautheron, D.C. (1975) Biochimie 57, 959–967
26 Ebel, R.E. and Lardy, H.A. (1975) J. Biol. Chem. 250, 191–196
27 Walseth, T.F. and Johnson, R.A. (1979) Biochim. Biophys. Acta 562, 11–31
28 Pullman, M.E. (1967) Methods Enzymol. 10, 57–71
29 Penin, F., Godinot, C., Comte, J. and Gautheron, D.C. (1982) Biochim. Biophys. Acta 679, 198–209
30 Godinot, C., Di Pietro, A. and Gautheron, D.C. (1975) FEBS Lett. 60, 250–255
31 Pedersen, P.L., Schwerzmann, K. and Cintrón, N. (1981) Curr. Top. Bioenerg. 11, 149–199
32 Klein, G., Satre, M., Dianoux, A.C. and Vignais, P.V. (1980) Biochemistry 19, 2919–2925
33 Klein, G., Lunardi, J. and Vignais, P.V. (1981) Biochim. Biophys. Acta 636, 185–192
34 Harris, D.A., Dall-Larsen, T. and Klungsoyr, L. (1981) Biochim. Biophys. Acta 635, 412–428
35 Grubmeyer, C., Cross, R.L. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12092–12100
36 Schuster, S.M., Ebel, R.E. and Lardy, H.A. (1975) J. Biol. Chem. 250, 7848–7853
37 Roux, B., Fellous, G. and Godinot, C. (1984) Biochemistry 23, 534–537
38 Baubichon, H., Di Pietro, A., Godinot, C. and Gautheron, D.C. (1982) FEBS Lett. 137, 261–264
39 Kozlov, I.A. and Vulfson, E.N. (1985) FEBS Lett. 182, 425–428
40 Penefsky, H.S. and Grubmeyer, C. (1984) in H^+-ATPase (ATPsynthase): Structure, Function, Biogenesis. The F_0F_1 complex of coupling membranes (Papa, S., Altendorf, K., Ernster, L. and Packer, L., eds.), pp. 195–204, ICSU Press and Adriatica Editrice, Bari