Journal of Bioenergetics and Biomembranes, Vol. 15, No. 6, 1983

Effect of the Protonmotive Force on ATP-Linked Processes and Mobilization of the Bound Natural ATPase Inhibitor in Beef Heart Submitochondrial Particles

Gérard Klein[1] and Pierre V. Vignais[1]

Received March 3, 1983; revised June 6, 1983

Abstract

In an attempt to determine whether the natural ATPase inhibitor (IF_1) plays a role in oxidative phosphorylation, the time course of ATP synthesis and ATP hydrolysis in inside-out submitochondrial particles from beef heart mitochondria either possessing IF_1 (Mg-ATP particles) or devoid of IF_1 (AS particles) was investigated and compared to movements of IF_1, as assessed by an isotopic assay. The responses of the above reactions to preincubation of the particles in aerobiosis with NADH or succinate were as follows: (1) The few seconds lag that preceded the steady-rate phase of ATP synthesis was shortened and even abolished both in Mg-ATP particles and AS particles. The rate of ATP synthesis in the steady state was independent of the length of the lag. (2) ATPase was slowly activated, maximal activation being obtained after a 50-min preincubation; there was no direct link between the development of the protonmotive force (maximal within 1 sec) and ATPase activation. (3) Bound IF_1 was slowly released; the release of bound IF_1 as a function of the preincubation period was parallel to the enhancement of ATPase activity; the maximal amount of IF_1 released was a small fraction of the total IF_1 bound to the particles (less than 20%). (4) The double reciprocal plots of the rates of ATP and ITP hydrolysis vs. substrate concentrations that were curvilinear in the absence of preincubation with a respiratory substrate became linear after aerobic preincubation with the substrate. The data conclusively show that only ATPase activity in submitochondrial particles is correlated with the release of IF_1, and that the total extent of IF_1 release induced by respiration is limited. On the other hand, the kinetics of ATPase in control and activated particles are consistent with the existence of two conformations of the membrane-bound F_1-ATPase, directed to ATP synthesis or ATP hydrolysis and distinguishable by their affinity for IF_1.

Key Words: Natural ATPase inhibitor; protonmotive force; mitochondrial ATPase.

[1]Laboratoire de Biochimie (CNRS/ERA 903 et INSERM U.191), Département de Recherche Fondamentale, Centre d'Etudes Nucléaires, 85X, 38041 Grenoble cedex, France.

0145-479X/83/1200-0347$03.00/0

Introduction

The beef heart ATPase inhibitor[2] IF_1 (Pullman and Monroy, 1963) is a small heat-stable protein of M_r 9578 (Frangione *et al.*, 1981) that binds to the β subunit of the F_1 sector of the ATPase complex (Klein *et al.*, 1980, 1981). This protein was called ATPase inhibitor because it inhibits ATP hydrolysis by mitochondrial ATPase, and also ATP-driven coupled reactions (Pullman and Monroy, 1963; Horstman and Racker, 1970; Asami *et al.*, 1970). Since the reaction catalyzed by mitochondrial ATPase is reversible, one might expect that IF_1 could inhibit ATP synthesis as well, and therefore play a regulatory function in oxidative phosphorylation. Experimental data along this line have been reported by Ernster *et al.* (1973), Van de Stadt *et al.* (1973), and Van de Stadt and Van Dam (1974) who showed that the establishment of a protonmotive force induces a transition of the hydrolytic activity of the bound ATPase complex from a latent state to an active state. On the other hand, in oxidative phosphorylation catalyzed by sonic particles, a lag phase was detected, which is shortened in particles that had developed a protonmotive force upon respiration prior to addition of ADP; this was explained by the release of bound IF_1; it was inferred that the rate of oxidative phosphorylation is regulated by the reversible binding of IF_1 to F_1 (Harris *et al.*, 1979; Gomez-Puyou *et al.*, 1979). Experiments aimed at tracing the bound and free IF_1 were recently reported by Schwerzmann and Pedersen (1981) and by Dreyfus *et al.* (1981). By means of radioactively labeled IF_1, Klein *et al.* (1981) have shown in preliminary experiments that some bound IF_1 is released to the medium when sonic particles are energized by respiration. This work has been pursued, and the effect of bound IF_1 on different reactions of the oxidative phosphorylation mechanism has been examined. The data reported in the present paper show that although a correlation can be established between IF_1 binding or IF_1 release with respect to sonic particles, and changes in ATPase activities, the total extents of these changes are very small indeed; their physiological significance is discussed in the context of a putative role of IF_1 as a regulatory ligand in oxidative phosphorylation.

[2]Abbreviations. $\Delta\tilde{\mu}H^+$: protonmotive force; ΔpH: pH gradient; $\Delta\psi$: membrane potential; FCCP: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops: 3-(*N*-morpholino)propane sulfonic acid; Ap_5A: P^1,P^5-di(adenosine-5')-pentaphosphate; sonic particles: submitochondrial particles obtained by sonic irradiation of beef heart mitochondria; Mg-ATP particles: sonic particles prepared in the presence of Mg-ATP; AS particles: sonic particles prepared in the presence of ammonia and filtrated on Sephadex G50; F_1: beef heart mitochondrial coupling factor; IF_1: beef heart ATPase inhibitor protein, called also natural protein inhibitor; PITC: phenylisothiocyanate; [^{14}C]PTC-IF_1: natural ATPase inhibitor radiolabeled by [^{14}C]PITC.

Experimental Procedure

Materials

Luciferin–luciferase "Lumit® PM" was obtained from Sempa, Paris, France. P^1,P^5-di(adenosine-5')-pentaphosphate (Ap_5A) was from Boehringer, and [^{14}C]phenylisothiocyanate (PITC) (10.2 mCi/mmol) from Amersham. 3,3'-Dipropylthiodicarbocyanine was kindly provided by Dr. A.S. Waggoner (Amherst College, Amherst, Massachusetts).

Methods

Biological Preparations. Beef heart mitochondria (Smith, 1967), AS particles (Racker and Horstman, 1967), Mg-ATP particles (Löw and Vallin, 1963), F_1-ATPase (Knowles and Penefsky, 1972), and IF_1 (Horstman and Racker, 1970) were prepared according to published methods. IF_1 was chemically radiolabeled with [^{14}C]PITC as described by Klein *et al.* (1980). In routine preparations, an average of 1 mol of [^{14}C]PITC was incorporated per mole of IF_1, resulting in a fully active [^{14}C]PTC-IF_1 with a specific radioactivity of 23×10^9 dpm/mmol.

Measurement of $\Delta\psi$ in Sonic Particles. The $\Delta\psi$ component of the protonmotive force in Mg-ATP particles was monitored by the fluorescent dye 3,3'-dipropylthiodicarbocyanine (Sims *et al.*, 1974; Waggoner *et al.*, 1977) as described by Villiers *et al.* (1979). In brief, the Mg-ATP particles (0.2 mg) were added to 3 ml of a medium containing 225 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM Mops, pH 6.5, 1.6 μM rotenone, and 3 μM dipropylthiodicarbocyanine. Particles were left in contact with the medium at room temperature for 2 min before addition of the substrate. Succinate was added at a final concentration of 3 mM. Excitation wavelength was set at 622 nm and emission wavelength at 670 nm.

ATPase Activity Assay. Routinely ATPase activity of Mg-ATP particles was measured at 28°C in a medium made of 0.05 M Tris-SO_4, pH 8.0, 4 mM phosphoenolpyruvate (tricyclohexylammonium salt), 40 μg/ml pyruvate kinase, 3 mM $MgCl_2$, 3 mM ATP, and 2 μM FCCP, by assay of released P_i (Fiske and SubbaRow, 1925). Alternatively, ATPase was determined by spectrophotometry using a reaction coupled to NADH oxidation in the presence of a regenerating system for ATP, as described by Chang and Penefsky (1973).

ATP Synthetase and ATPase Assays by the Luciferin–Luciferase System. The assay was adapted from Lundin *et al.* (1976). Mg-ATP particles were suspended in 20 ml of medium containing 0.23 M sucrose, 10 mM KCl, 3 mM $MgCl_2$, and 10 mM Tris-SO_4, pH 7.5 (medium A) and

supplemented with 2 μM Ap_5A as an inhibitor of adenylate kinase in the absence or presence of 10 mM succinate. The assay was carried out at 28°C with an Aminco-Chance spectrophotometer with the measuring light turned off. The assay cuvette had a light path of 1 cm; it was covered with an aluminum foil on three unused faces to provide maximal light reflection. The open face was directed toward the photomultiplier. A sample of 450 μl of the particle suspension in medium A was introduced into the cuvette, followed by 50 μl of the luciferin–luciferase mixture. ATP synthesis was initiated by addition of 50 μl of 20 mM KP_i and 50 μM ADP, final pH 7.5, and ATP hydrolysis by addition of 50 μl of 16 μM ATP and 16 μM FCCP. As the amounts of ADP or ATP were far from being saturating, the assay was considered only as a qualitative test for monitoring the ATP synthetase or ATPase activities.

Assay of [^{14}C]PTC-IF$_1$ Release. This assay was performed with Mg-ATP particles that had exchanged their bound IF_1 against added radiolabeled IF_1. Mg-ATP particles (40 mg) were incubated at 28°C for 15 min with [^{14}C]PTC-IF_1 (0.4 mg) in 40 ml of a medium containing 0.25 M sucrose, 10 mM Mops, pH 6.5, 0.5 mM ATP, and 1 mM $MgCl_2$. After incubation, the particles were sedimented by centrifugation for 15 min at 25,000 *g*. The walls of the tubes were rinsed with cold water, and the pellets resuspended in 40 ml of medium A. In routine experiments, the amount of [^{14}C]PTC-IF_1 bound per milligram of Mg-ATP particles ranged from 150 to 200 pmol. Binding of [^{14}C]PTC-IF_1 occurred most probably by exchange with the bound IF_1 that was released to the medium, since the inhibitory activity of the supernatant after sedimentation of the particles by centrifugation did not vary during the course of incorporation of [^{14}C]PTC-IF_1. The [^{14}C]PTC-IF_1-loaded particles were then incubated in the absence or presence of 10 mM succinate, under constant stirring in order to keep the solution aerobic. Aliquots were withdrawn to measure ATPase activity; other aliquots were centrifuged to sediment the particles, and [^{14}C]PTC-IF_1 was determined both in the supernatant and in the pellet.

Assay of [^{14}C]PTC-IF$_1$ Binding. As particles (that are devoid of bound IF_1) were used. Increasing amounts of [^{14}C]PTC-IF_1 (up to 2.5 μg) were added to a fixed concentration of AS particles (2 mg protein) in 2 ml of the same medium as that used for [^{14}C]PTC-IF_1 exchange. After a 15-min incubation, aliquots were withdrawn for an ATPase assay and the remaining was centrifuged for 15 min at 25,000 *g*. The walls of the tubes were rinsed with cold water, and the pellets were dissolved in formamide at 180°C for radioactivity counting. The same assay was performed with particles denaturated by a 5-min treatment at 100°C. The ^{14}C radioactivity remaining bound to the denatured particles was considered as nonspecific and subtracted from the ^{14}C radioactivity bound to control AS particles.

Protein Determination. Protein was determined as described by Bradford (1976) for soluble F_1 and IF_1 and by the Biuret method for submitochondrial particles (Gornall *et al.*, 1949).

Results

Competence of Mg-ATP Particles to Develop a Protonmotive Force

The particles used in the present work were routinely checked for their ability to develop a protonmotive force upon addition of a respiratory substrate. Fluorescence changes of dipropylthiodicarbocyanine were chosen as an indicator of $\Delta\psi$ (see Methods). As shown previously, under similar conditions of medium and with similar particles, the protonmotive force developed was in the range of 180–200 mV, half of which corresponded to $\Delta\psi$ and the other half of ΔpH (Villiers *et al.*, 1979). On addition of succinate, the fluorescence intensity of dipropylthiodicarbocyanine increased abruptly to reach a plateau, in agreement with preceding data from this laboratory (Villiers *et al.*, 1979). This effect is opposite to that found in intact mitochondria because of the reverse polarity of the membrane in sonic particles as compared to intact mitochondria. Oligomycin, which is known to plug the F_0 sectors devoid of F_1, did not enhance the extent of the signal corresponding to $\Delta\psi$, suggesting that the F_0 units in the particle preparations used were all connected to F_1.

Effect of Preincubation of Mg-ATP Particles or AS Particles with Succinate or NADH on the Time Course of ATP Synthesis

The kinetics of ATP synthesis by Mg-ATP particles assayed by the luciferin–luciferase system are shown in Fig. 1A. For the particles not preincubated with a respiratory substrate, the steady-rate phase of ATP synthesis was preceded by a lag of about 40 sec; the length of the lag was markedly shortened by preincubation with succinate (Fig. 1). The same observation holds for NADH. The longer the preincubation period, the shorter the lag phase. After a 10-min preincubation, the lag had virtually disappeared. Whereas the lag preceding the steady-state phosphorylation was altered by preincubation with succinate or NADH, the rate of the steady-state phosphorylation itself was virtually unaffected.

Mg-ATP particles are characterized by a complete supply of bound IF_1. To assess the putative role of bound IF_1 in the lag phase preceding the steady-rate phase of ATP synthesis, a similar experiment was carried out with AS particles which are known to be devoid of IF_1. The phosphorylation kinetics with AS particles were virtually similar to those observed with

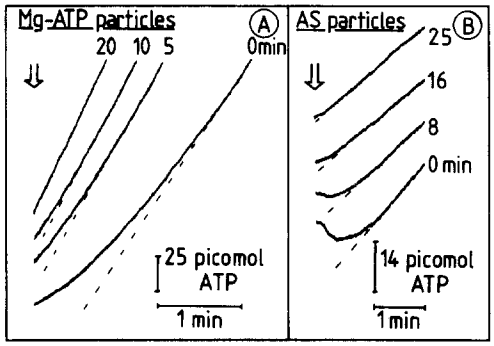

Fig. 1. ATP synthesis capacity of Mg-ATP particles and AS particles. (A) The ATP synthesis capacity of Mg-ATP particles was measured after a preincubation of 0, 5, 10, or 20 min in the presence of 10 mM succinate at 28°C. ATPase was assayed by the luciferin–luciferase technique as described under Methods. The assay was carried out with 0.02 mg of particles. ATP synthesis was initiated by addition of 50 μl of a mixture of 10 mM succinate, 20 mM KP_i, and 50 μM ADP, at the time indicated by the arrow (Fig. 1A). The steady-state phosphorylation rate was 6–7 nmol ATP synthesized per minute per milligram protein. Numbers beside the traces indicate the time of preincubation with succinate (min). (B) The ATP synthesis capacity of AS particles (0.05 mg) was measured after a preincubation of 0, 8, 16, or 25 min as described above for Mg-ATP particles. The steady-state phosphorylation rate was 0.3 nmol ATP synthesized per minute per milligram protein.

Mg-ATP particles, i.e., a lag followed by a steady-state phosphorylation (Fig. 1B). This finding casted some doubt about the idea that removal of bound IF_1 induced by the protonmotive force generated by respiration was responsible for the lag phase (Gomez-Puyou *et al.*, 1979).

Effect of Preincubation of Mg-ATP Particles with a Respiratory Substrate on the Time Course of ATP Hydrolysis

In the experiment of Fig. 2, Mg-ATP particles were incubated for various periods of time with NADH under constant aeration. Following preincubation, the particles were withdrawn and their ATPase activity with respect to a fixed concentration of ATP (3 mM) was determined by assay of P_i released (cf. Methods). Addition of FCCP in the ATPase assay medium was required to ensure that ATP hydrolysis was not rate-limited by the proton gradient generated by respiration. Preincubation with NADH for 30 min resulted in a threefold increase of the ATPase activity. In this experiments and

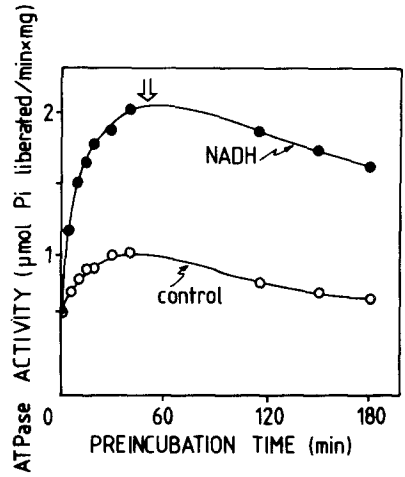

Fig. 2. Stimulation of the ATPase activity of Mg-ATP particles by respiration on NADH. Mg-ATP particles were diluted to 0.07 mg/ml in medium A in the absence (○) or in the presence (●) of 1.5 mM NADH at 28°C. 1-ml aliquots were taken as a function of the length of preincubation, and the ATPase activity of the particles was measured as P_i released (cf. Methods) in the presence of 3 mM ATP and 2 µM FCCP. The vertical arrow corresponds to NADH exhaustion by oxidation, as determined by NADH assay.

many others, the maximal ATPase activity attained by the preincubated Mg-ATP particles did not exceed 2 µmol of ATP hydrolyzed per minute per milligram protein, which is five times less than the ATPase activity found in particles totally depleted in IF_1 by alkaline treatment followed by sieve chromatography (AS particles).

The above experiment, based on a direct P_i assay on separated samples, was duplicated by a similar one where the ATPase activity was measured by the luciferin–luciferase assay. Although this latter method allowed continuous recording, its main drawback was that, for technical purpose, the ATP concentration to be used was far below saturation; as a result, the ATPase activity assayed was very low. This assay was therefore essentially qualitative; in spite of this, it provided interesting informations with respect to the time course of ATP hydrolysis. In the experiment of Fig. 3, the Mg-ATP particles were either preincubated or not preincubated with succinate. As shown in the traces, ATPase activity started soon after addition of ATP; the short lag (less than 10 sec) before ATPase activity became steady probably resulted from an artifact caused by mixing ATP with the particles. As in the experiment of Fig. 2, the ATPase of Mg-ATP particles in the experiment of Fig. 3 was maximally increased about fourfold following preincubation of the particles with succinate for 30 min.

In a separate experiment (data not shown), it was confirmed (Van de Stadt *et al.*, 1973) that preincubation of Mg-ATP particles with NADH and succinate, but not with ascorbate, enhanced ATPase activity significantly. Addition of rotenone and antimycin in the case of NADH oxidation, and antimycin in the case of succinate oxidation, prevented ATPase activation.

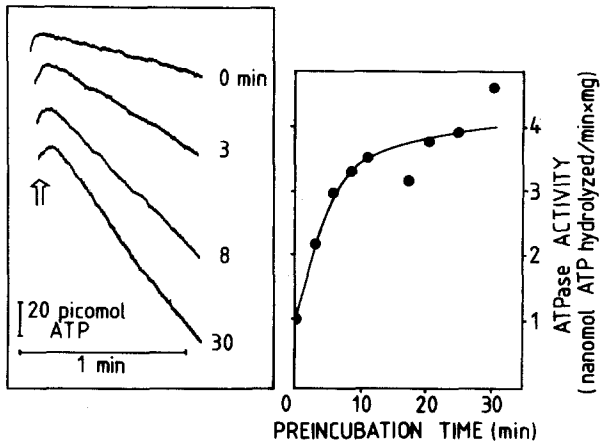

Fig. 3. Stimulation of the ATPase activity of Mg-ATP particles by respiration on succinate. Mg-ATP particles were diluted to a concentration of 0.04 mg protein/ml in 20 ml of medium A containing 10 mM succinate and 1.6 μM Ap_5A at 28°C. At different times, 0.45-ml aliquots were removed and added to a cuvette containing 50 μl luciferin–luciferase (see Methods) and 50 μl of a mixture of 16 μM ATP and 16 μM FCCP. ATP hydrolysis corresponded to a decrease in luminescence. The left part shows a few selected traces. Numbers beside the traces indicate the time of preincubation with succinate (0, 3, 8, and 30 min). In the right part, the increase in ATPase activity was plotted as a function of the period of preincubation.

Nigericin, which collapses ΔpH, and valinomycin, which collapses $\Delta\psi$, had no significant effect when added separately; however, when added together they prevented ATPase activation as did FCCP.

Binding of [^{14}C]PTC-IF$_1$ to AS Particles and Its Release from [^{14}C]PTC-IF$_1$ Preloaded Mg-ATP Particles upon Respiration

As shown in Fig. 4, incubation of AS particles with increasing concentrations of [^{14}C]PTC-IF$_1$ in the presence of Mg-ATP resulted in an increased binding of [^{14}C]PTC-IF$_1$ up to a plateau level of 250 pmol per milligram of particles. The fact that the amount of bound [^{14}C]PTC-IF$_1$ is equal to that of F$_1$ present in submitochondrial particles (Mitchell and Moyle, 1974; Ferguson *et al.*, 1976) is consistent with the idea that IF$_1$ binds to F$_1$ with a 1 to 1 stoichiometry (Klein *et al.*, 1980). Moreover, the K_d value relative to the binding of [^{14}C]PTC-IF$_1$ to AS particles, 25 nM, is very close to the K_i found previously for inhibition of F$_1$ by IF$_1$, namely 20 nM (Klein *et al.*, 1977). Binding of [^{14}C]PTC-IF$_1$ was accompanied by the concomitant inhibition of ATPase activity. Attempts to reverse binding and inhibition by initiation of respiration were unsuccessful. It was therefore decided to use Mg-ATP

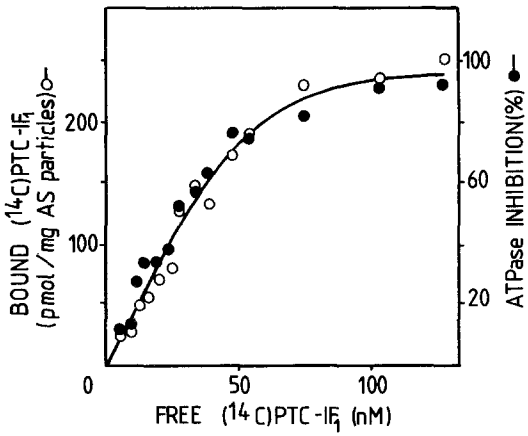

Fig. 4. Correlation between binding of [^{14}C]PTC-IF_1 to AS particles and inhibition of ATPase activity. AS particles were incubated with increasing concentrations of [^{14}C]PTC-IF_1 as described under Materials and Methods. Prior to centrifugation, aliquots were withdrawn to assay ATPase activity. The control ATPase activity (without added [^{14}C]PTC-IF_1) was 6 μmol P_i released/min × mg. The bound [^{14}C]PTC-IF_1 was measured in the pellet.

particles in which the bound IF_1 has been replaced by exchange with [^{14}C]PTC-IF_1 (see Methods). With these particles, we examined whether activation of the membrane-bound ATPase by respiration could be correlated with the release of bound radioactivity. This experiment was similar in principle to that described in Fig. 3, except that the Mg-ATP particles used in the present experiment were labeled by [^{14}C]PTC-IF_1. Following different periods of incubation, the particles were sedimented by centrifugation; the ATPase activity of the particles and the remaining bound [^{14}C]PTC-IF_1 were determined. Although the results were somewhat scattered, due to the limited release of [^{14}C]PTC-IF_1 and the slight increase in ATPase activity, the two events were consistently correlated. For example, after a 30-min incubation with succinate, the ATPase activity was raised from 0.30 to 0.80 μmol ATP hydrolyzed per minute per milligram protein, and the [^{14}C]PTC-IF_1 released was 17 pmol/mg protein. The released [^{14}C]PTC-IF_1 corresponded to about 7% of the total IF_1 binding capacity of the particles, based on a mean average of reported values of about 250 pmol of F_1/mg particle protein (Mitchell and Moyle, 1974; Ferguson *et al.*, 1976) and on the established IF_1/F_1 stoichiometry of 1 for complete ATPase inhibition (Klein *et al.*, 1980). Since the maximal ATPase activity that can be elicited by full release of IF_1 by alkaline treatment and Sephadex chromatography (AS particles) is roughly 10 μmol/min/mg particle protein (Racker and Horstman, 1967), it was inferred

that in untreated particles the ATPase activity was (10 − 0.30)/10 = 97% inhibited and that in succinate-treated particles ATPase inhibition was (10 − 0.80)/10 = 92%. Assuming that ATPase inhibition is related to binding of IF_1 to F_1, the difference between the two percentages, i.e. 5%, resulted from the amount of F_1 that had been freed from IF_1, i.e., 12.5 pmol. The percentage of IF_1 released, calculated by the enzymatic assay, 5%, was in good agreement with the percentage of IF_1 released, 7%, determined by the isotopic assay. After 10 min of preincubation, the percentages of IF_1 released, calculated either from the increase in ATPase activity or directly determined by the isotopic assay, were also very close and ranged from 2 to 3%. In the experiment of Fig. 2 (see above), it could be calculated that about 20% of the F_1 molecules had been activated after a 40-min preincubation with NADH; this was the maximal value found in our hands.

Effect of Preincubation of Mg-ATP Particles with a Respiratory Substrate on the Kinetic Parameters of the Membrane-Bound ATPase

In all the above experiments, the effect of succinate on ATPase activation of Mg-ATP particles was assayed with a fixed concentration of ATP. A similar activation experiment with succinate was carried out at different concentrations of ATP up to 3 mM; the results are illustrated by the double reciprocal plots in Fig. 5A. In the case of control Mg-ATP particles the plots of ATP hydrolysis were not linear; a similar situation was reported for the

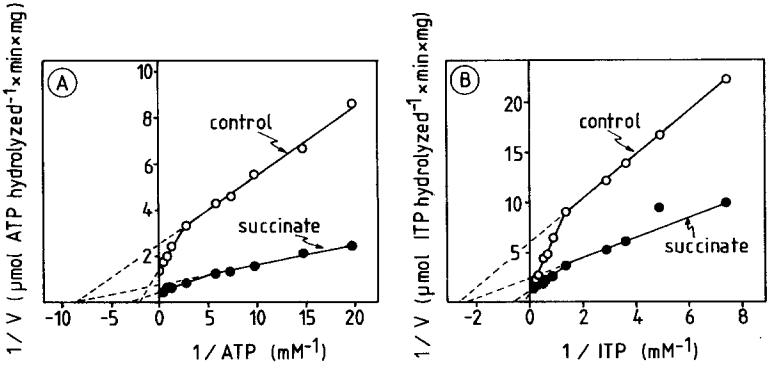

Fig. 5. Double reciprocal plots of ATPase and ITPase activity of Mg-ATP particles incubated or not incubated with succinate. Mg-ATP particles were suspended at a concentration of 0.07 mg/ml in medium A in the absence (○) or in the presence (●) of 10 mM succinate. After a 30-min preincubation at 28°C, 1-ml fractions were transferred to 2 ml of the ATPase assay medium described by Chang and Penefsky (1973). Hydrolytic activity was determined for different concentrations of ATP (left pannel) and ITP (right pannel) up to 3 mM. Kinetic data are presented as the reciprocal of the velocity $(\mu mol/min \times mg)^{-1}$ vs. the reciprocal of substrate concentration (mM^{-1}).

Table I. Kinetic Parameters of Membrane-Bound ATPase and ITPase in Mg-ATP Particles[a]

Respiratory substrate in the preincubation medium	Nucleotides used	V_{max} (μmol/min × mg)		K_M (mM)	
		V_1	V_2	K_1	K_2
No respiratory substrate	ATP	0.35	0.70	0.1	0.5
	ITP	0.15	0.90	0.3	6.7
Succinate	ATP	1.00	1.50	0.1	0.2
	ITP	0.35	0.90	0.4	2.0

[a]The V_{max} and K_M values are derived from the plots of Fig. 5. V_1 and K_1 on the one hand, and V_2 and K_2, on the other, refer to values of V_{max} and K_M obtained by extrapolation of the plots corresponding to low and high concentrations of nucleotides, respectively.

isolated F_1 (Schuster *et al.*, 1975; Pedersen, 1976). Upon addition and oxidation of succinate, not only the ATPase activity was enhanced, but a Michaelian type of kinetics was obtained. The kinetic parameters corresponding to the two curves in Fig. 5 for control Mg-ATP particles and Mg-ATP particles incubated with succinate are listed in Table I. The shift from curvilinear to linear kinetics upon preincubation of the Mg-ATP particles with a respiratory substrate can be compared to the shift from linear to curvilinear kinetics by interaction of IF_1 with isolated beef heart F_1 (Krull and Schuster, 1981). Although the experimental approaches were different, a similarity can also be found between the kinetics reported in the present paper with beef heart Mg-ATP particles and those reported by Cintron *et al.* (1982) with inner membrane vesicles of rat liver, loaded with IF_1. In our case, the extrapolated reciprocal plots of the rate vs. ATP concentration for the control assay (segment of the curve corresponding to high concentrations of ATP) and for the succinate assay intersected between the vertical and the base axes. Assuming that the lower ATPase activity in the control assay as compared to the succinate assay was due to inhibition by bound IF_1, the graphical representation in Fig. 5A was typical of a mixed type inhibition. When ATP was replaced by ITP, a similar activation of hydrolytic activity by succinate was observed, but the reciprocal plots for the segments of the curves concerning high ITP concentrations intersected on the vertical axis, indicating a competitive type of inhibition (Fig. 5B).

Discussion

Is IF_1 a Strict ATPase Inhibitor or Does It Inhibit Both ATPase and ATP Synthetase Activities?

The regulatory function of IF_1 in the process of oxidative phosphorylation is still a matter of debate. After the discovery of IF_1, it was first proposed that

the function of IF_1 was unidirectional, being limited to the inhibition of the hydrolytic activity of the mitochondrial ATPase complex (Pullman and Monroy, 1963) and ATP-driven reactions (Asami *et al.*, 1970; Ernster *et al.*, 1973). A further step was the discovery that the ATPase activity of sonic particles was enhanced by oxidation of NADH or succinate and inhibited by a high ATP/ADP ratio (Van de Stadt *et al.*, 1973); this was interpreted as being due to the release of IF_1 from respiring particles and to an increased affinity of IF_1 for the particles at high concentrations of ATP; however, no binding studies were carried out. In a more recent work, it was proposed that, since F_1 in the ATPase complex has the ability to both synthetize and hydrolyze ATP, IF_1 must have the ability when it binds to the ATPase complex to inhibit both ATP synthesis and ATP hydrolysis (Gomez-Puyou *et al.*, 1979; Harris *et al.*, 1979). Release of bound IF_1 was believed to depend on the protonmotive force generated by respiration. In this case again, no direct binding data were reported. In a preliminary report from this laboratory (Klein *et al.*, 1981), it was shown by means of a radiolabeled derivative of IF_1 that indeed the increase of ATPase activity of Mg-ATP particles upon oxidation of NADH was accompanied by a release of bound IF_1. Two other papers have been concerned with the mobilization of IF_1 upon energization of sonic particles. Schwerzmann and Pedersen (1981) also found that energized sonic particles do release a fraction of bound IF_1 to the medium; on the other hand, Dreyfus *et al.* (1981), using ^{125}I-labeled antibodies, showed that, in energized sonic particles, the mobilized IF_1 remained bound to sonic particles, being, however, more accessible to antibodies, as if a shift from a cryptic state to a more accessible state had occurred.

In the experiment described in the present paper, the steady-state ATP synthetase activity of Mg-ATP particles was not affected by the aerobic preincubation, in agreement with Schwerzmann and Pedersen (1981). On the other hand the lag phase in phosphorylation, previously reported by Harris *et al.* (1979), Gomez-Puyou *et al.* (1979), and Schwerzmann and Pedersen (1981) was abolished by aerobic preincubation of the Mg-ATP particles with a respiratory substrate; however, the aerobic preincubation had to last for at least 10 min, a period of time markedly greater than that required for the development of the protonmotive force (less than 1 sec). Finally, two types of particles differing by their IF_1 content, Mg-ATP particles that are fully equipped with IF_1 and AS particles that are devoid of IF_1, exhibited the same lag period prior to the steady-rate phase of ATP synthesis. All these data taken together strongly suggest that the lag phase in ATP synthesis is not related to the presence of bound IF_1.

A long preincubation with a respiratory substrate (30–50 min) was required for maximal enhancement of the ATPase activity. During this preincubation, IF_1 was continuously released; the amount of IF_1 released,

measured by an isotopic assay, was consistent with the unmasked ATPase activity. It must be borne in mind, however, that the IF_1 release was slow and its maximal extent limited; for example, no more than 20% of the bound IF_1 was released after a 40-min preincubation. A similar low percentage of ATPase activation could be calculated from the data reported by Schwerzmann and Pedersen (1981) under somewhat different conditions.

The very limited effect of respiration on ATPase activation and IF_1 release raises the question of whether IF_1 has a regulatory function in the process of oxidative phosphorylation. If IF_1 does control oxidative phosphorylation, what could be the reason why a clear demonstration of such a function could not be achieved? A possible explanation is that the sonic particles routinely used are not the appropriate material for tracing a regulatory effect of IF_1 on ATP synthesis or ATP hydrolysis. On the other hand, assuming that the experiments reported with the Mg-ATP particles reflect the situation in intact mitochondria, one is led to conclude that there is no marked regulatory function of IF_1 in oxidative phosphorylation. One may therefore ask why IF_1 binds so firmly and specifically to the catalytic subunit of F_1, namely the β subunit (Klein *et al.*, 1980, 1981). A plausible hypothesis suggested by Pedersen *et al.* (1981) quoting Trumpower is that IF_1 could freeze the ATPase activity of the F_1 factor, when the ATPase is synthesized and assembled in the cell.

Does IF_1 Trigger a Transition Between Different Conformational States of F_1?

As shown in the above section, aerobic preincubation of submitochondrial particles with an oxidizable substrate resulted in some unmasking of ATPase activity which was concomitant with the release of IF_1. Examination of the kinetics of ATPase in control particles and preincubated particles showed differences which might be related to some regulatory mechanism. Whereas the rates of ATP and ITP hydrolysis by control particles vs. the concentration of ATP and ITP yielded curvilinear plots in the Lineweaver–Burk representation, the unmasked ATPase and ITPase in preincubated particles gave almost linear plots (Fig. 5). This situation is the mirror image of that described by Krull and Schuster (1981) and Cintron *et al.* (1982) who found that incubation of soluble or membrane-bound F_1-ATPase with IF_1 resulted in the transformation of the linear Lineweaver–Burk plots for the ATPase reaction into curvilinear plots. Since the effects are reciprocal, it is plausible to admit that the causes are reciprocal, i.e., that the control Mg-ATP particles are inhibited by IF_1 and upon incubation IF_1 is released with a concomitant increase in ATPase activity. In the case of ITP hydrolysis, there was a common intercept of the plots on the ordinate for nonactivated and

activated ITPase, suggesting that nonactivated ITPase in control particles was controlled by an endogenous competitive inhibitor. The situation was more complex when ITP was replaced by ATP, since the masking of ATPase in control particles resembled a mixed type of inhibition. In the following discussion, we shall consider the simple case of ITP hydrolysis. The curvilinearity of the plots of the masked ITPase in control particles may correspond to the sum of two separate kinetic processes for ITP hydrolysis, differing by their K_d values for ITP and the binding affinity of IF_1. One of the two processes would predominate at low concentrations of ITP. By extrapolation of the portion of the plots corresponding to low ITP concentrations, one finds that the extrapolated slope intercepts the abscissa virtually at the same point as the extrapolated plots for the activated enzyme, which suggests a noncompetitive inhibition for ITP hydrolysis in control particles at low or moderate concentrations of ITP. On the other hand, at high ITP concentrations, as mentioned above, abolition of inhibition would reflect a second kinetic process involving competitive inhibition for ITP hydrolysis. These two processes are best explained by two conformational states of F_1, which are in equilibrium and are characterized by quite different affinities for ITP, namely a high-affinity conformation □ and a low-affinity conformation o (see scheme of Fig. 6). Only the low-affinity conformation is considered to be recognized by IF_1. At low ITP concentrations, the maximum velocity (derived by extrapolation)

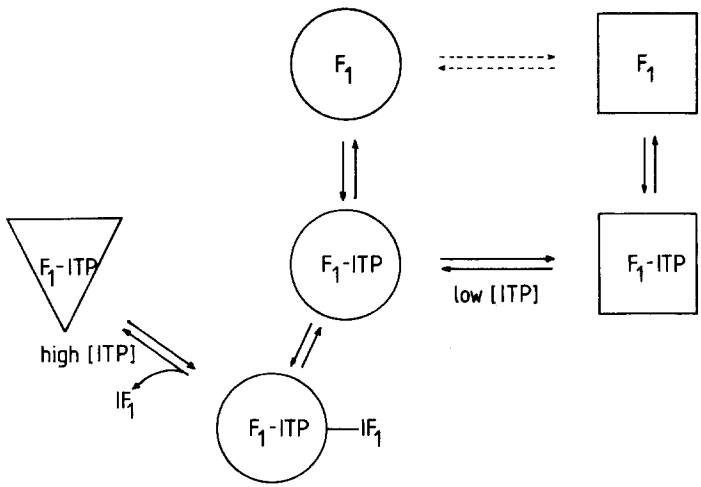

Fig. 6. Hypothetical scheme depicting the inhibition mechanism by IF_1. The scheme assumes an IF_1-triggered transition between two main conformations of F_1, one □ exhibiting a high affinity for ITP (or ATP), the other o exhibiting a low affinity. A supplementary conformation ▽ is postulated and supposed to accumulate at very high concentrations of ITP (or ATP). For more details, see Discussion.

concerns essentially the high-affinity conformation □. On the other hand, at high ITP concentrations, the maximum velocity (intercept of the ordinate) is the sum of the maximal velocities catalyzed by the two conformations. As shown in the scheme of Fig. 6, the equilibrium between the two conformations is shifted towards the low-affinity conformation ○ by binding of IF_1 to this conformation at low or intermediate concentrations of ITP (<0.5 mM) in control particles. When the particles are preincubated with succinate, F_1 takes the high-affinity conformation □ and IF_1 is released. Binding of IF_1 to F_1 in conformation ○ decreases the concentration of F_1 in conformation □; this explains the apparent noncompetitive inhibition which characterizes ITP hydrolysis in control particles as compared to ITP hydrolysis in preincubated particles. On the other hand, to explain why, at high concentrations of ITP (>1 mM), the rates of ITP hydrolysis in control and preincubated particles tend to the same value, one has to admit that IF_1 bound to F_1 in conformation ○ is released by high concentrations of ITP, most likely by indirect interaction involving a further conformational change of F_1 into ▽, due possibly to the binding of ITP to a regulatory site with low affinity for ITP. The same basic reasoning holds when ATP is used as substrate instead of ITP; the slight difference in kinetics reflects a more complex situation for ATP than for ITP hydrolysis, with respect to the regulatory control by ATP concentration (Schuster *et al.*, 1975). In both cases, however, a number of turnovers of F_1 are required for the binding of IF_1 (Gomez-Fernandez and Harris, 1978); as illustrated in the scheme of Fig. 6, this would mean that the transition between the two main conformations □ and ○ is slow in the absence of a hydrolyzable substrate, and rapid in its presence. Although at present evidence is lacking, it may be suggested that ATP is hydrolyzed by F_1 in conformation □ and synthesized by F_1 in conformation ○. Ernster *et al.* (1979) also proposed that IF_1 binding to F_1 depended on the conformation of F_1. They postulated that F_1 incubated with ATP and Mg^{++} assumes a conformation of high affinity for IF_1 and that in respiring submitochondrial particles, the protonmotive force results in a conformation of low affinity for IF_1. In other words, in contrast with our scheme, they implied a hydrolytic state of F_1 which firmly binds IF_1 and a synthetic state which loosely binds IF_1; their scheme, however, could not explain both the noncompetitive type of inhibition exhibited by IF_1 at low concentrations of ATP and the competitive type at high concentrations of ATP.

Acknowledgment

This investigation was supported in part by research grants from the Fondation pour la Recherche Médicale.

References

Asami, K., Juntti, K., and Ernster, L. (1970). *Biochim. Biophys. Acta* **205,** 307–311.

Bradford, M. M. (1976). *Anal. Biochem.* **72,** 248–254.

Chang, T.-M., and Penefsky, H. S. (1973). *J. Biol. Chem.* **248,** 2746–2754.

Cintron, N. M., Hullihen, J., Schwerzmann, K., and Pedersen, P. L. (1982). *Biochemistry* **21,** 1878–1885.

Dreyfus, G., Gomez-Puyou, A., and Tuena de Gomez-Puyou, M. (1981). *Biochem. Biophys. Res. Commun.* **100,** 400–406.

Ernster, L., Juntti, K., and Asami, K. (1973). *J. Bioenerg.* **4,** 149–159.

Ernster, L., Carlsson, C., Hundal, T., and Nordenbrand, K. (1979). *Methods Enzymol.* **55,** 399–407.

Ferguson, S. J., Lloyd, W. J., and Radda, G. K. (1976). *Biochem. J.* **159,** 347–353.

Fiske, C. H., and SubbaRow, Y. (1925). *J. Biol. Chem.* **66,** 375–400.

Frangione, B., Rosenwasser, E., Penefsky, H. S., and Pullman, M. E. (1981). *Proc. Natl. Acad. Sci. USA* **78,** 7403–7407.

Gomez-Fernandez, J. C., and Harris, D. A. (1978). *Biochem. J.* **176,** 967–975.

Gomez-Puyou, A., Tuena de Gomez-Puyou, M., and Ernster, L. (1979). *Biochim. Biophys. Acta* **547,** 252–257.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). *J. Biol. Chem.* **177,** 751–766.

Harris, D. A., Von Tscharner, V., and Radda, G. K. (1979). *Biochim. Biophys. Acta* **548,** 72–84.

Horstman, L. L., and Racker, E. (1970). *J. Biol. Chem.* **245,** 1336–1344.

Klein, G., Satre, M., and Vignais, P. V. (1977). *FEBS Lett.* **84,** 129–134.

Klein, G., Satre, M., Dianoux, A. C., and Vignais, P. V. (1980). *Biochemistry* **19,** 2919–2925.

Klein, G., Satre, M., Dianoux, A. C., and Vignais, P. V. (1981). *Biochemistry* **20,** 1339–1344.

Knowles, A. F., and Penefsky, H. S. (1972). *J. Biol. Chem.* **247,** 6617–6623.

Krull, K. W., and Schuster, S. M. (1981). *Biochemistry* **20,** 1592–1598.

Löw, H., and Vallin, I. (1963). *Biochim. Biophys. Acta* **69,** 361–374.

Lundin, A., Rickardsson, A., and Thore, A. (1976). *Anal. Biochem.* **75,** 611–620.

Mitchell, P., and Moyle, J. (1974). *Biochem. Soc. Spec. Publ.* **4,** 91–111.

Pedersen, P. L. (1976). *J. Biol. Chem.* **251,** 934–940.

Pedersen, P. L., Schwerzmann, K., and Cintron, N. (1981). *Curr. Top. Bioenerg.* **11,** 149–199.

Pullman, M. E., and Monroy, G. C. (1963). *J. Biol. Chem.* **238,** 3762–3769.

Racker, E., and Horstman, L. L. (1967). *J. Biol. Chem.* **242,** 2547–2551.

Schuster, S. M., Ebel, R. E., and Lardy, H. A. (1975). *J. Biol. Chem.* **250,** 7848–7853.

Schwerzmann, K., and Pedersen, P. L. (1981). *Biochemistry* **20,** 6305–6311.

Sims, P. J., Waggoner, A. S., Wang, C. H., and Hoffman, J. F. (1974). *Biochemistry* **13,** 3315–3330.

Smith, A. L. (1967). *Methods Enzymol.* **10,** 81–86.

Van de Stadt, R. J., De Boer, B. L., and Van Dam, K. (1973). *Biochim. Biophys. Acta* **292,** 338–349.

Van de Stadt, R. J., and Van Dam, K. (1974). *Biochim. Biophys. Acta* **347,** 240–252.

Villiers, C., Michejda, J. W., Block, M., Lauquin, G. J. M., and Vignais, P. V. (1979). *Biochim. Biophys. Acta* **546,** 157–170.

Waggoner, A. S., Wang, C. H., and Tolles, R. L. (1977). *J. Membr. Biol.* **33,** 109–140.