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EFFECT OF THE NATURAL ATPase INHIBITOR ON THE BINDING OF ADENINE NUCLEOTIDES AND INORGANIC PHOSPHATE TO MITOCHONDRIAL F_1-ATPase

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(1) Incubation of the beef heart mitochondrial ATPase, F_1 with Mg-ATP was required for the binding of the natural inhibitor, IF_1, to F_1 to form the inactive F_1-IF_1 complex. When F_1 was incubated in the presence of [^{14}C]ATP and $MgCl_2$, about 2 mol ^{14}C-labeled adenine nucleotides were found to bind per mol of F_1; the bound ^{14}C-labeled nucleotides consisted of [^{14}C]ADP arising from [^{14}C]ATP hydrolysis and [^{14}C]ATP. The ^{14}C-labeled nucleotide binding was not prevented by IF_1. These data are in agreement with the idea that the formation of the F_1-IF_1 complex requires an appropriate conformation of F_1. (2) The ^{14}C-labeled adenine nucleotides bound to F_1 following preincubation of F_1 with Mg-[^{14}C]ATP could be exchanged with added [^{3}H]ADP or [^{3}H]ATP. No exchange occurred between added [^{3}H]ADP or [^{3}H]ATP and the ^{14}C-labeled adenine nucleotides bound to the F_1-IF_1 complex. These data suggest that the conformation of F_1 in the isolated F_1-IF_1 complex is further modified in such a way that the bound ^{14}C-labeled nucleotides are no longer available for exchange. (3) $^{32}P_i$ was able to bind to isolated F_1 with a stoichiometry of about 1 mol of P_i per mol of F_1 (Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899). There was no binding of $^{32}P_i$ to the F_1-IF_1 complex. Thus, not only the nucleotides sites, but also the P_i site, are masked from interaction with external ligands in the isolated F_1-IF_1 complex.

Introduction

Free or membrane-bound mitochondrial ATPase (F_1) possesses tight and loose binding sites for ADP and ATP [1]. It also contains a site which binds inorganic phosphate (P_i) with moderate affinity [2]. The tightly bound ADP and ATP in the ATPase of inside-out submitochondrial particles can be readily exchanged with added ADP or ATP, provided the mitochondrial membrane is energized [3,4]; exchange of the tightly bound nucleotides in F_1 is possible when the pH of the medium is slightly acidic [5]. The natural ATPase inhibitor, IF_1, is a small peptide of M_r lower than 10000 [6] whose binding to F_1 requires a preincubation step with F_1 and Mg^{2+} [7]. The energized exchange of adenine nucleotides in submitochondrial particles is inhibited by IF_1 [3]. To our knowledge, there were no data on the effect of IF_1 on the exchange of bound adenine nucleotides by isolated F_1. The present investigation was carried out with F_1 isolated from beef heart mitochondria; its main purpose was to determine to what extent the capacity for exchange of bound adenine nucleotides was modified in the F_1-IF_1 complex. The effect of IF_1 on the binding of P_i to isolated F_1 was also explored.

Abbreviation: Mops, 3-(*N*-morpholino)propanesulfonic acid.

Materials and Methods

Materials. [U-^{14}C]ATP (300 mCi/mmol) and carrier-free $^{32}P_i$ were obtained from the Commis-

sariat à l'Energie Atomique, Saclay, France. [2-^{3}H]-ADP (16.5 Ci/mmol) and [2-^{3}H]ATP (24 Ci/mmol) were obtained from Amersham.

Preparation of F_1 and IF_1. Beef heart F_1 was purified and stored as an ammonium sulfate suspension as described by Knowles and Penefsky [8] and beef heart IF_1 by the technique of Horstman and Racker [7], as modified by Kagawa [9]. Before use, the F_1 suspension was centrifuged and the pellet was solubilized in 250 mM sucrose/10 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), pH 6.5; this solution was desalted by the elution-centrifugation method described by Penefsky [2]. After desalting, the F_1 preparation contained 0.98 ± 0.22 mol ATP/mol F_1 and 1.67 ± 0.36 mol ADP/mol F_1 (four determinations), as measured in perchloric acid extracts after neutralization [10].

Binding of IF_1 to F_1 and loading of F_1 with ^{14}C-labeled adenine nucleotides. In routine experiments, F_1 (80–100 μg) was incubated with increasing amounts of IF_1 (up to 9 μg) in 100 μl of a medium containing 0.25 M sucrose/10 mM Mops/1 mM $MgCl_2$/0.5 mM [^{14}C]ATP at pH 6.5 for 30 min. This step allowed the binding of IF_1 to F_1 and the concomitant loading of F_1 with ^{14}C-labeled adenine nucleotides. The ^{14}C-labeled adenine nucleotides bound to F_1 or the F_1-IF_1 complex were then separated from free [^{14}C]ATP by a centrifugation-filtration technique [2], using 1-ml syringes filled with Sephadex G-50 (fine) equilibrated in 0.25 M sucrose/10 mM Mops, pH 6.5 with either 1 mM $MgCl_2$ or 2 mM EDTA. To assess the tight or loose character of the binding of ^{14}C-labeled adenine nucleotides to F_1, the ^{14}C-labeled adenine nucleotide-loaded enzyme was precipitated four times by saturated ammonium sulfate [11]. The remaining bound nucleotides are refered to as tightly bound nucleotides. In routine assays, F_1 was loaded with roughly 2 mol of ^{14}C-labeled adenine nucleotides per mol of enzyme. Out of these 2 mol of bound nucleotides, 1.5 mol could be removed by ammonium sulfate precipitation and are therefore considered as loosely bound nucleotides; the remaining bound nucleotides are the tightly bound.

Exchange of loaded ^{14}C-labeled adenine nucleotides with added ADP. Unlabeled or [^{3}H]ADP was added to F_1 or the F_1-IF_1 complex previously loaded with ^{14}C-labeled adenine nucleotides, and incubated for 30 min at room temperature; it was checked that full exchange was attained after 20–25 min of incubation. After the centrifugation-filtration step as described above, the filtrate containing the newly bound ^{3}H radioactivity and the remaining ^{14}C radioactivity was assayed for ATPase activity, protein content, ^{14}C and ^{3}H radioactivities.

Binding of $^{32}P_i$. $^{32}P_i$ was added to F_1 or the F_1-IF_1 complex previously preincubated with unlabeled Mg-ATP. After a 30 min-incubation at room temperature, free and bound $^{32}P_i$ were separated by centrifugation-filtration. The filtrate was assayed for ATPase activity, protein content and $^{32}P_i$ radioactivity.

ATPase assay. ATPase activity was assayed with a regenerating system containing 50 mM Tris-SO_4, pH 8.0/10 mM ATP/5 mM $MgCl_2$/40 μg/ml pyruvate kinase/4 mM phosphoenol pyruvate. The incubation was carried out at 30°C for 10 min and stopped by addition of cold perchloric acid. The phosphate released by ATP hydrolysis was measured in the perchloric extracts [12].

Protein concentration. Protein concentration was measured by the method of Bradford [13] using Coomassie blue G 250. Bovine serum albumin was used as a standard.

Results

Effect of IF_1 on the binding of ^{14}C-labeled adenine nucleotides to F_1

An acidic pH and Mg-ATP are required for optimal binding of IF_1 to F_1 [6,7,14]. Thus, the medium used for nucleotide binding was supplemented with 1 mM $MgCl_2$, and its pH was adjusted to 6.5. In the experiment illustrated in Fig. 1 starting from 0.5 mM [^{14}C]ATP, the amount of bound ^{14}C-labeled adenine nucleotides recovered after passage through a Sephadex column was 2.1 mol/mol F_1 (0.6 mol [^{14}C]ATP and 1.5 mol [^{14}C]ADP). In four experiments, the amount of bound ^{14}C-labeled nucleotides ranged between 2 and 2.5 mol/mol F_1; the percentage of bound [^{14}C]ADP was between 70 and 80%, the remaining radioactivity belonging to [^{14}C]ATP.

As shown in Fig. 1, ^{14}C-labeled adenine nucleotide binding was not modified by inclusion of IF_1 in the incubation medium, even at concentrations of IF_1 that fully inhibit the ATPase activity of F_1.

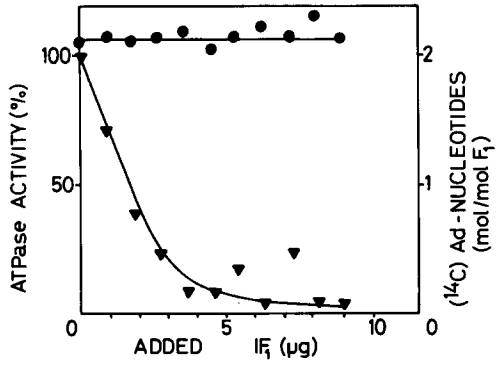

Fig. 1. Effect of IF_1 on the binding of [^{14}C]ATP to F_1. F_1 was incubated as described under Materials and Methods with increasing amounts of IF_1 in a medium containing 1 mM $MgCl_2$ and 0.5 mM [^{14}C]ATP, pH 6.5. After an incubation of 30 min, the mixture was filtrated through a syringe filled with Sephadex G-50, and the filtrate assayed for protein, ^{14}C radioactivity and ATPase activity. The figure shows the ATPase inhibition (▼——▼) and the bound ^{14}C-labeled nucleotides (●——●) vs. the IF_1 concentrations. Ad-Nucleotides, adenine nucleotides.

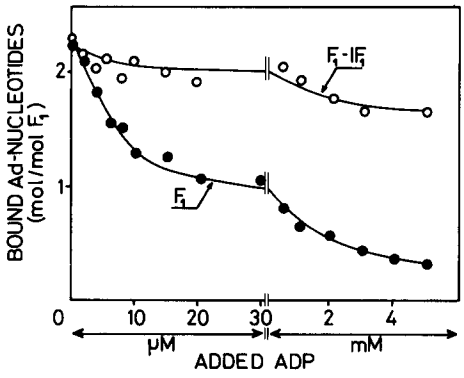

Fig. 2. Chase of F_1 bound ^{14}C-labeled nucleotides by ADP. F_1 was preincubated at pH 6.5 with 1 mM $MgCl_2$ and 0.5 mM [^{14}C]ATP in the presence (○——○) or in the absence (●——●) of IF_1. F_1 and the F_1-IF_1 complex with bound ^{14}C-labeled adenine nucleotides were recovered by Sephadex filtration, and incubated with increasing concentrations of ADP in the presence of 1 mM $MgCl_2$. After a 30 min incubation, the mixtures were passed through Sephadex, and the filtrates, corresponding to F_1 or F_1-IF_1, were assayed for bound ^{14}C radioactivity. Ad-Nucleotides, adenine nucleotides.

Effect of IF_1 on the exchange of F_1-bound ^{14}C-labeled adenine nucleotides with added [^{3}H]ADP

The aim of the experiment illustrated in Fig. 2 was to compare the effect of increasing concentrations of added ADP on the removal of bound ^{14}C-labeled adenine nucleotides from F_1 and the F_1-IF_1 complex that were previously loaded with ^{14}C-labeled nucleotides as described above. In the case of F_1, out of the 2.3 mol of bound ^{14}C-labeled nucleotides per mol of the enzyme, (about 2/3 of [^{14}C]ADP and 1/3 of [^{14}C]ATP), 1.3 mol were readily displaced by concentrations of ADP as low as 20 μM, half displacement being obtained with 6 μM ADP; bound [^{14}C]ADP and [^{14}C]ATP were displaced to the same extent. The remaining bound ^{14}C-labeled nucleotides (about 1 mol/mol F_1) were removed only by millimolar concentrations of ADP, the half maximum effect being observed at 1.6 mM ADP.

The same displacement experiment, conducted with a F_1-IF_1 complex in which 76% of the ATPase activity of F_1 was inhibited (Fig. 2), indicated an inhibition of the ADP-induced release of the bound ^{14}C radioactivity of about 70% at any concentrations of ADP between 20 μM and 4 mM. Similar results were obtained when ADP was replaced by ATP, as the displacing reagent (not shown).

To characterize more accurately the effect of IF_1, double-labeling experiments were carried out at

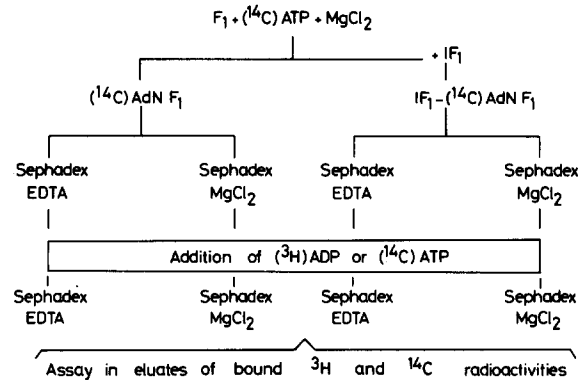

Fig. 3. Assay of the exchange of F_1-bound ^{14}C-labeled adenine nucleotides with [^{3}H]ADP or [^{3}H]ATP in the presence of EDTA or $MgCl_2$. Note that the binding of IF_1 to F_1 requires $MgCl_2$ and ATP. The F_1-IF_1 complex is stable, even when treated further with EDTA.

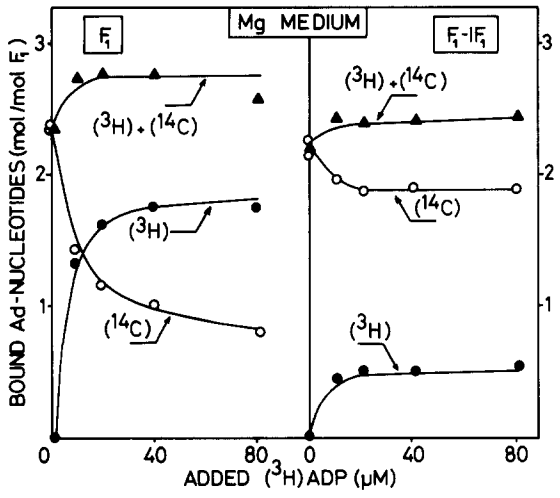

Fig. 4. Exchange of bound ^{14}C-labeled adenine nucleotides with increasing concentrations of added [^3H]ADP in a $MgCl_2$ medium. Effect of IF_1. The conditions are those of Figs. 2 and 3. IF_1 was added at a concentration inhibiting 90% of the F_1-ATPase activity. Ad-Nucleotides, adenine nucleotides.

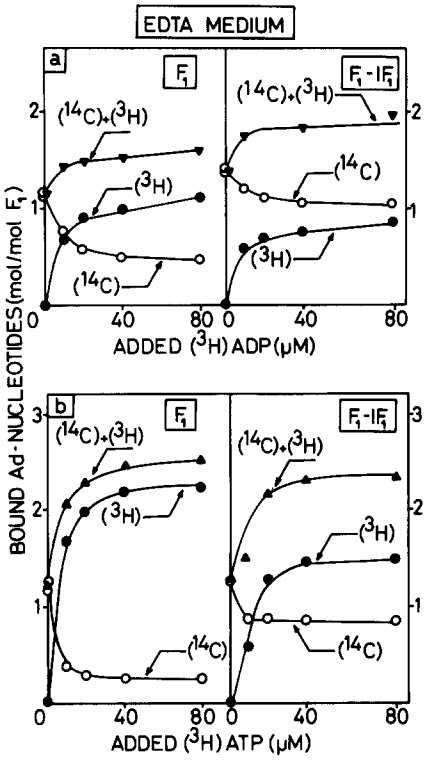

Fig. 5. Exchange of bound ^{14}C-labeled adenine nucleotides with increasing concentrations of added [^3H]ADP or [^3H]-ATP in an EDTA medium. Effect of IF_1. Same conditions as in Fig. 4, except that the F_1-ATPase activity was 80% inhibited. Ad-Nucleotides, adenine nucleotides.

different concentrations of added [^3H]ADP or [^3H]ATP, either in the presence of $MgCl_2$ or EDTA, as outlined in the scheme of Fig. 3. The data of Fig. 4 ($MgCl_2$ medium) show that, in the case of F_1, in the absence of IF_1, the newly bound ^3H radioactivity increased in proportion with the added [^3H]ADP, whereas the previously bound ^{14}C radioactivity decreased. The sum of the bound ^3H and ^{14}C radioactivities remained virtually constant over a large range of [^3H]ADP concentrations, but was somewhat higher than the amount of bound ^{14}C radioactivity in the absence of [^3H]ADP. This indicated that binding of added [^3H]ADP occurred mainly by exchange with previously bound ^{14}C-labeled adenine nucleotides; only a small part of the binding of [^3H]ADP corresponded to a net uptake. Similar data were obtained when [^3H]ADP was replaced by [^3H]ATP. The nucleotide exchange capacity was much lower in the F_1-IF_1 complex than in F_1; in contrast, the binding of [^3H]ADP or [^3H]ATP corresponding to net uptake was not inhibited in the F_1-IF_1 complex.

The data in Fig. 5 concern the exchange of F_1-bound [^{14}C]ADP and [^{14}C]ATP with [^3H]ADP or [^3H]ATP in an EDTA medium. They differ somewhat from those obtained with the $MgCl_2$ medium (Fig. 4), although here again a striking decrease of nucleotide exchange in the F_1-IF_1 complex could also be demonstrated. Upon treatment by EDTA, F_1 preloaded in the presence of $MgCl_2$ with 2 mol of ^{14}C-labeled adenine nucleotides per mol of enzyme lost one of the two bound ^{14}C-labeled nucleotides. Upon further addition of [^3H]ADP (Fig. 5a), the remaining ^{14}C-labeled nucleotide was released in exchange for [^3H]ADP; this exchange was inhibited in the F_1-IF_1 complex. When [^3H]ATP was added instead of [^3H]ADP in the same EDTA medium (Fig. 5b), both a net uptake of [^3H]ATP (1 mol/mol of F_1) and an exchange of [^3H]ATP against bound ^{14}C-labeled nucleotides were observed. The exchange of bound ^{14}C-labeled adenine nucleotides against

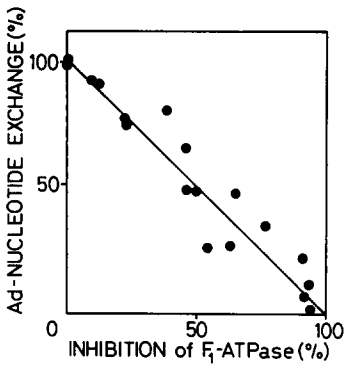

Fig. 6. Correlation between nucleotide exchange capacity and ATPase inhibition by IF_1. The conditions were the same as in Figs. 2 and 4. The ATPase activity was determined on filtrates of Sephadex column (cf. Materials and Methods). Ad-Nucleotide, adenine nucleotide.

added [^{3}H]ATP was again inhibited in the F_1-IF_1 complex; however the [^{3}H]ATP uptake remained similar to that found for F_1. One may wonder why the F_1-IF_1 complex is still able to bind 1 mol of [^{3}H]ATP per mol of complex, while being unable to exchange the already bound ^{14}C-labeled nucleotide. In fact, the newly bound [^{3}H]ATP appears to replace the bound nucleotide that was lost upon treatment of F_1-IF_1 by EDTA (see above).

Experiments of the same type as those described in Figs. 4 and 5 were carried out at different concentrations of added [^{3}H]ATP with preparations of F_1 loaded with IF_1 to different extents, and the capacity of exchange of the bound ^{14}C-labeled adenine nucleotides with added [^{3}H]ADP in these preparations was examined as a function of the inhibition of the ATPase activity. As shown in Fig. 6, the exchange capacity of the bound nucleotides decreased in proportion with the ATPase activity of F_1 inhibited by IF_1. Since a direct and linear relationship between the amount of IF_1 bound to isolated F_1 and the inhibition of the F_1-ATPase activity has been clearly established [15], it follows that the binding of IF_1 to F_1 is responsible for the loss of the ability to exchange F_1-bound nucleotides. It must be noted that inhibition of the F_1-ATPase activity by IF_1 after preincubation with Mg-ATP was always refered to control F_1 preincubated with Mg-ATP, in order to eliminate any interference due to the known inhibition of F_1 preincubated with Mg-ATP [5].

Effect of IF_1 on the binding of P_i to F_1

Penefsky [2] briefly reported that binding of IF_1 to F_1 interfered with the binding of P_i to F_1, with half inhibition corresponding to the binding of 0.5 mol of IF_1 per mol of F_1. The following experiment was carried out to quantify the inhibitory effect of IF_1 on P_i binding to F_1. In the experiment of Fig. 7, F_1 was preincubated for 30 min at pH 6.5, at room temperature, with ATP, $MgCl_2$ and an amount of IF_1 inhibiting 80% of the F_1-ATPase activity. Then ATP was removed by centrifugation-filtration on Sephadex as described under Materials and Methods, and the binding capacity of F_1 in the F_1-IF_1 complex for increasing concentrations of $^{32}P_i$ was assayed. $^{32}P_i$ binding was clearly inhibited in a noncompetitive manner. As shown in Fig. 8, the binding of $^{32}P_i$ to F_1 was inversely related to the inhibition of the F_1-ATPase activity by IF_1 (which reflects the extent of binding of IF_1 to F_1). Under our experimental conditions, one P_i binding site per F_1 was observed. When F_1 was 100% inhibited by IF_1, the binding capacity of F_1 for P_i was totally lost.

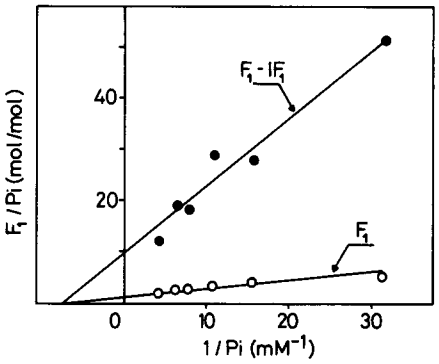

Fig. 7. Binding of $^{32}P_i$ to F_1 and F_1-IF_1 complex. F_1 was incubated with 0.5 mM ATP and 1 mM $MgCl_2$ in the presence (●——●) or in the absence (○——○) of IF_1 as described under Materials and Methods. The IF_1-F_1 complex and F_1 were recovered by filtration on Sephadex and then incubated with $^{32}P_i$ for 30 min. The mixture was filtered through a Sephadex G-50 (fine) column, and the eluate tested for protein content and $^{32}P_i$ radioactivity. F_1/P_i is plotted as a function of $1/P_i$.

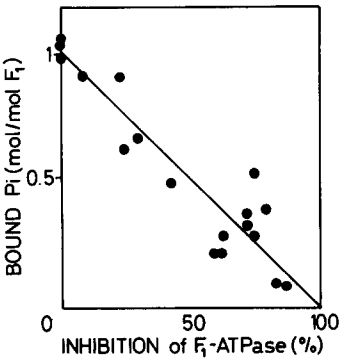

Fig. 8. Correlation between $^{32}P_i$ binding and F_1 inhibition. F_1 was incubated with 0.5 mM ATP and 1 mM $MgCl_2$ and increasing amounts of IF_1 to produce different levels of inhibition ranging from 0 to 90%. The mixtures were passed through Sephadex (cf. Materials and Methods) and the filtrates were incubated with $^{32}P_i$. After a 30 min incubation, the filtrates were again passed through Sephadex. The eluates were assayed for protein content, ATPase activity and $^{32}P_i$ radioactivity. The ratio of $^{32}P_i$ bound per F_1 is plotted as a function of ATPase inhibition.

Discussion

This paper is mainly concerned with the effect of IF_1 on the interaction of adenine nucleotides with isolated F_1. In this respect, two types of effects have to be distinguished according to whether one considers the binding to F_1 of adenine nucleotides arising from hydrolysis of added Mg-ATP in the presence of IF_1 or the exchange of added ADP with bound adenine nucleotides in F_1 or the F_1-IF_1 complex.

Addition of [^{14}C]ATP in the presence of $MgCl_2$ resulted in the binding of about 2 mol of ^{14}C-labeled nucleotides per mol of F_1, consisting of [^{14}C]ADP arising from [^{14}C]ATP hydrolysis and [^{14}C]ATP itself. Whether ^{14}C-labeled nucleotide binding corresponds to net uptake or to exchange with previously bound nucleotides is not known. The important point is that the binding of IF_1 to F_1, concomitant with [^{14}C]ATP hydrolysis, did not alter the extent of ^{14}C-labeled nucleotide binding to F_1. The critical event which triggers the binding of IF_1 by F_1 in the presence of Mg-ATP, and therefore determines the inhibition of the enzymic activity of F_1 is linked to hydrolysis of Mg-ATP [5,7,14]. The requirement for bound ATP to induce the binding of IF_1 to F_1 has been further stressed by the demonstration that phosphorylation of F_1-bound ADP into ATP increases the inhibitory effect of IF_1 on the ATPase activity of F_1 [16]. Probably upon Mg-ATP binding followed by ATP hydrolysis, F_1 assumes a new conformation state, F_1^*, that facilitates the binding of IF_1.

In contrast to the absence of an effect of IF_1 on the binding to F_1 of ^{14}C-labeled nucleotides arising from [^{14}C]ATP added together with IF_1, the exchange of these bound ^{14}C-labeled nucleotides with secondarily added [^{3}H]ADP or [^{3}H]ATP is inhibited in the F_1-IF_1 complex. These data obtained with isolated F_1 extend those reported by Harris et al. [3] on the effect of IF_1 on the exchange of bound nucleotides in submitochondrial particles. In the case of submitochondrial particles, two types of adenine nucleotide exchange, energy-dependent and energy-independent, were distinguished [3]; only the 'energized' exchange was inhibited by IF_1; however interpretation of the data obtained with submitochondrial particles calls for some reservations, due to the complexity of the system. For example, upon energization by oxidation of NADH, the exchange capacity of particles rich in IF_1 remained stable, whereas that of particles depleted in IF_1 was decreased; in fact just the opposite finding would have been expected.

As postulated above and in agreement with Gomez-Fernandez and Harris [17], the hydrolysis of Mg-ATP by F_1 may result in a new conformation, F_1^*, which is more appropriate for the binding of IF_1. The F_1^* conformation could also occur in energized submitochondrial particles. Once IF_1 has bound to F_1^*, the exchange of F_1-bound nucleotides in the $^*F_1$-IF_1 complex with added [^{3}H]ADP is inhibited. The simplest interpretation is that the nucleotide binding sites could be blocked by IF_1. However, as shown below, the F_1-IF_1 interaction is probably more complicated. At this point, it is interesting to mention that IF_1 is practically irreversibly bound to isolated F_1 (unpublished observations); this is not the case for IF_1 in submitochondrial particles [18–20]. On this basis, one may imagine that the F_1^*-IF_1 complex made from isolated F_1^* is readily shifted towards a stable irreversible $^{**}F_1$-IF_1 conformation. The $^{**}F_1$-IF_1 conformation

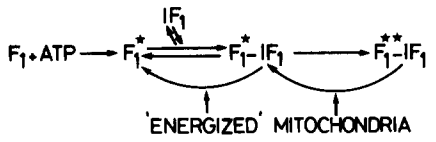

Fig. 9. Conformational states assumed by F_1 in the IF_1-F_1 complex. Upon ATP hydrolysis, the conformation of F_1 is modified to give $^*F_1$, a conformation that is able to bind reversibly IF_1. The conformation of $^*F_1$ in $^*F_1$-IF_1 is further modified to give the $^{**}F_1$-IF_1 complex; the return to the $^*F_1$-IF_1 state is not possible with isolated F_1, but is supposed to be possible in the case of membrane-bound F_1 by energization of the mitochondria as indicated by arrows.

could also occur in submitochondrial particles, but, in this case, upon energization of the membrane, the $^*F_1$-IF_1 conformation could be recovered with possible release of bound IF_1 (cf. scheme of Fig. 9). This might explain why, with isolated F_1, a strictly linear relationship exists between the adenine nucleotide exchange and the binding of IF_1 (this paper), whereas, in the case of membrane-bound F_1, the inhibition of the adenine nucleotide exchange is not proportional to the binding of IF_1 [3]. The assumed irreversible binding of IF_1 to $^{**}F_1$ in the isolated $^{**}F_1$-IF_1 complex could also explain some puzzling results obtained in preliminary photolabeling experiments, dealing with the covalent photolabeling of F_1 or the F_1-IF_1 complex by *N*-4-azido-2-nitrophenyl-aminobutyryl ADP, a photoactivable derivative of ADP. As previously reported [21], this photolabel, upon photoirradiation, inactivates F_1; full inactivation corresponded to the covalent binding of 2 mol of the azido derivative per mol of F_1, on both the α and β subunits of F_1 [22]. The surprising finding was that IF_1 added to F_1 prior to photoirradiation in the presence of of the photolabel was able to decrease the amount of covalently bound photolabel, both in the α and β subunits [23]. In other words, the extent of photolabeling is lower in the F_1-IF_1 complex than in F_1 in spite of the fact that photolabeling is a covalent process; this corroborates the idea of an irreversible change of conformation of F_1 into F_1^{**} in the F_1-IF_1 complex; further, the data suggest that this conformational change is not restricted to the β subunit of F_1 to which IF_1 binds [15,24], and which also possesses the catalytic site [25], but also to the adjacent α subunit [26].

Not only does IF_1 inhibit adenine nucleotide exchange by F_1; it also prevents the binding of P_i. The inhibition of P_i binding is linearly related to the binding of IF_1 to F_1, as was the case for the inhibition of adenine nucleotide exchange. The data on P_i binding and adenine nucleotide exchange taken together favor the idea that the binding of IF_1 to F_1 induces a new conformational state of F_1, in which the adenine nucleotide and P_i binding sites are not any longer accessible to their respective ligands.

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