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# The Native $F_0F_1$-Inhibitor Protein Complex from Beef Heart Mitochondria and its Reconstitution in Liposomes

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A functional $F_0F_1$ ATP synthase that contains the endogenous inhibitor protein ($F_0F_1I$) was isolated by the use of two combined techniques [Adolfsen, R., McClung, J.A., and Moudrianakis, E. N. (1975). *Biochemistry* **14,** 1727–1735; Dreyfus, G., Celis, H., and Ramirez, J. (1984). *Anal. Biochem.* **142,** 215–220]. The preparation is composed of 18 subunits as judged by SDS-PAGE. A steady-state kinetic analysis of the latent ATP synthase complex at various concentrations of ATP showed a $V_{max}$ of 1.28 $\mu$mol min$^{-1}$ mg$^{-1}$, whereas the $V_{max}$ of the complex without the inhibitor was 8.3 $\mu$mol min$^{-1}$ mg$^{-1}$. In contrast, the $K_m$ for Mg-ATP of $F_0F_1I$ was 148 $\mu$M, comparable to the $K_m$ value of 142 $\mu$M of the $F_0F_1$ complex devoid of $IF_1$. The hydrolytic activity of the $F_0F_1I$ increased severalfold by incubation at 60°C at pH 6.8, reaching a maximal ATPase activity of 9.5 $\mu$mol min$^{-1}$ mg$^{-1}$; at pH 9.0 a rapid increase in the specific activity of hydrolysis was followed by a sharp drop in activity. The latent ATP synthase was reconstituted into liposomes by means of a column filtration method. The proteoliposomes showed ATP-Pi exchange activity which responded to phosphate concentration and was sensitive to energy transfer inhibitors like oligomycin and the uncoupler *p*-trifluoromethoxyphenylhydrazone.

**KEY WORDS:** $F_0F_1$ ATP synthase; inhibitor protein; protein reconstitution.

## INTRODUCTION

The ATP synthases from cytoplasmic, thylakoidal, and mitochondrial inner membranes synthesize ATP coupled to an electrochemical gradient of protons generated by the photosynthetic or respiratory electron transport chain. These enzymes vary in complexity; nevertheless, their basic structure is conserved among a wide variety of phylogenetically diverse species. In all cases, the enzyme is arranged in two domains (for review see Futai *et al.*, 1989 and Pedersen and Amzel, 1993). The catalytic portion of the enzyme ($F_1$), consists of five subunits $\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, which can be detached from membranes as a soluble ATPase. The integral membrane sector, $F_0$, consists of three subunits *a*, *b*, and *c* and functions as a proton pathway in ATP synthesis and hydrolysis. The ATP synthase complex catalyzes the hydrolysis and synthesis of ATP coupled to proton translocation and is thought to be regulated by an endogenous polypeptide referred to as the natural inhibitor protein. (Pullman and Monroy, 1963; for reviews, see Pedersen *et al.*, 1981, Gautheron and Godinot, 1988, and Harris and Das, 1991). The interest in obtaining an ATP synthase preparation that contains a functional inhibitor protein is that this will enable us to ask direct questions on the role of $IF_1$ on energy conservation and synthesis of ATP.

Here we have made use of two combined techniques (Adolfsen *et al.*, 1975 and Dreyfus *et al.*, 1984) to obtain a latent ATP synthase-inhibitor protein complex ($F_0F_1I$) with catalytic properties similar to the latent $F_1$ ATPase previously characterized by Feinstein and Moudrianakis (1984). The presence of the natural inhibitor protein was demonstrated by

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kinetic and by thermal and ion strength-induced increase in ATPase activity. The properties of this preparation contrast to those of ATP synthase, devoid of the regulating peptide, prepared as described by Dreyfus *et al.* (1984). The native ATP synthase complex reported here was reconstituted into phospholipid vesicles; the reconstituted enzyme carried out ATP-Pi exchange. This activity responds to inorganic phosphate and bovine serum albumin and is highly sensitive to the uncoupler *p*-trifluoromethoxyphenylhydrazone (FCCP) and oligomycin.

## MATERIALS AND METHODS

### Preparation of Submitochondrial Particles

Bovine heart mitochondria were prepared as described by Löw and Vallin (1963); "heavy mitochondria" were stored at −75°C until used. Submitochondrial particles devoid of inhibitor protein ($IF_1$) were prepared by resuspending mitochondria in a buffer containing 250 mM KCl, 75 mM sucrose, 30 mM Tris-$SO_4$ (pH 8.0), 2 mM EDTA, and processed as described by Klein *et al.* (1982). Submitochondrial particles with a high content of inhibitor protein (Mg-ATP particles) were prepared by resuspending mitochondria in a buffer containing 250 mM sucrose, 6 mM ATP, and 6 mM Mg-acetate and processed according to Lee and Ernster (1967). Submitochondrial particles were stored in liquid nitrogen until used.

### Isolation and Reconstitution of the Mitochondrial $F_0F_1$ ATP Synthase

*Isolation Procedure*

In this work two different $F_0F_1$ ATP synthase preparations were isolated. $F_0F_1$ ATP synthase devoid of $IF_1$ and a latent form containing the endogenous inhibitor protein were obtained according to the method of Dreyfus *et al.* (1984), except that the solubilization of the $F_0F_1$ ATP synthase was performed with the nonionic detergent *n*-dodecyl-β-D-maltoside. For the isolation of the latent $F_0F_1$ ATP synthase, 13.5 mg of Mg-ATP submitochondrial particles were diluted to a concentration of 3 mg/ml in a buffer containing 150 mM sucrose, 20 mM Mes-Tris (pH 6.8), and 100 mM ADP. A volume of *n*-dodecyl-β-D-maltoside was added to the submitochondrial particles suspension at a final concentration of 3.0 mg of detergent/mg of protein and incubated for 30 min at 4°C. The mixture was centrifuged 60 min at 100,000 × *g*. The pellet was resuspended in the same volume of the above-mentioned buffer and lauryl maltoside added at the same concentration. The supernatants were pooled and applied to a Sepharose-AH column (1 × 5 cm) equilibrated with a buffer containing 20 mM Mes-Tris (pH 6.8), 2 mM EDTA, 10 mM sodium cholate, 5 mM ADP, and 3 mg/ml asolectin; fractions of 5 ml were collected. The column was washed with 40 ml of the same buffer before eluting the retained $F_0F_1$ ATP synthase. Elution was carried out by the addition of 12.5 ml of the same buffer supplemented with 1 M KCl. The fractions were tested for ATPase activity and protein concentration, and concentrated by the addition of one volume of saturated ammonium sulfate (pH 6.8). After incubation at 4°C for 30 min, the sample was centrifuged 15 min at 20,500 × *g*. The pellet was resuspended in a buffer containing 250 mM sucrose, 20 mM Mes-Tris (pH 6.8), 2 mM EDTA, 2 mM sodium cholate, 100 μM ADP, and 30 mg/ml asolectin and stored in liquid nitrogen until used.

*Reconstitution into Liposomes*

The ATP synthase complex was reconstituted using a variation of the elution–centrifugation method described by Garrett and Penefsky (1975) as modified by Vázquez-Contreras *et al.* (unpublished results). Freshly thawed (100–150 μg) $F_0F_1$ complex was applied to a 1-ml syringe containing Sephadex G50 fine previously equilibrated with Mes-Tris 20 mM (pH 6.8) and 2 mM EDTA. The column was centrifuged for 1 min at 3000 rpm in a Sorvall RT 6000B bench top centrifuge. Protein yield was ca. 90%.

*Hydrolysis of ATP*

ATP hydrolysis of the $F_0F_1$ and $F_0F_1I$ ATP synthase (10–20 μg) was measured in 350 μl of a medium containing 100 mM Tris-$SO_4$ (pH 7.8), 3 mM ATP, 5 mM MgCl, 4 mM phosphoenolpyruvate, and 32 μg of pyruvate kinase. Incubation was carried out at 30°C for 10 min and the reaction was stopped by the addition of 100 μl trichloroacetic acid (6% final concentration). The amount of inorganic phosphate liberated was determined according to Fiske and SubbaRow (1925) or to

Lanzetta (1979) for a higher sensitivity. ATP hydrolysis was also determined spectrophotometrically as described by Pullman *et al.* (1960).

*ATP-Pi Exchange Reaction*

The proteoliposomes (100 $\mu$g) of protein were diluted to 300 $\mu$l in 250 mM sucrose. The reaction was started by the addition of 300 $\mu$l of a medium containing 50 mM Tris acetate (pH 7.5), 2 or 15 mM $H_3PO_4$ (pH 7.2 with Tris base), 5 mM ADP, 3 mM ATP, 10 mM $MgSO_4$, 250 mM sucrose, and $^{32}Pi$ ($10^7$ cpm); in some experiments 1 mg/ml of bovine serum albumin was included. The reaction was incubated for 30 min at 30°C and stopped by the addition of TCA to 6% final concentration. The determination of $^{32}Pi$ incorporated into ATP was performed as described by Dreyfus (1985).

**Other Procedures**

Immunoblotting was carried out after separating proteins by SDS-PAGE, and the proteins were electrophoretically transferred to nitrocellulose for 5 h (Towbin *et al.*, 1979). The sheets were blocked with 3% gelatin in $H_2O$ for 1 h. Anti-$IF_1$ antiserum prepared as described by Dreyfus *et al.* (1981) was used at a 1:1000 dilution 1% gelatin in 100 mM Tris-HCl (pH 7.5), 0.9% NaCl, and 0.1% (vol/vol) Tween 20. The nitrocellulose sheets were then processed by using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, California).

Polyacrylamide gel electrophoresis was carried out in a gradient (10–23%) in the presence of 0.1% sodium dodecyl sulfate according to Laemmli (1970). The gels were scanned in an Ultroscan XL, LKB Bromma, Sweden using a Gel Scan Program from Pharmacia.

Protein determinations of the membrane fractions were measured as reported by Gornall *et al.* (1949) and according to Smith *et al.* (1985) for the soluble material. Bovine serum albumin was used as standard with both methods. Sepharose-AH, Sephadex G50 fine, phospholipids (phosphatidylcholine, type II-S from soybean), oligomycin, FCCP, dicyclohexylcarbodiimide (DCCD), and *n*-dodecyl-$\beta$-D-maltoside were from Sigma Chemical Co. Electron micrographs were taken with a Jeol microscope model 1200 EXII operated at 80 kV. Specimens were negatively stained with 2% (w/v) potassium phosphotungstate, pH 6.8.

## RESULTS

### Structural and Kinetic Characterization of $F_0F_1$ and $F_0F_1I$ Preparations

*Structural Characterization*

A latent $F_0F_1I$ ATP synthase was isolated from latent submitochondrial particles that had the ATP synthase in complex with the inhibitor protein (MgATP submitochondrial particles). The methodology employed was based on the ability of the $F_1$ ATPase to bind to Sepharose-AH (Tuena de Gómez-Puyou and Gómez-Puyou, 1977; Dreyfus *et al.*, 1984). The procedure developed renders a latent complex containing the natural ATPase inhibitor ($IF_1$). After solubilization with *n*-dodecyl-$\beta$-D-maltoside under acidic conditions in the presence of ADP, the enzyme was purified by Sepharose-AH chromatography. Under these conditions the interaction between $F_1$ and $IF_1$ is preserved. Figure 1 shows the electrophoretic pattern of $F_0F_1I$, the $F_0F_1$ ATP synthase, and $IF_1$. The ATP synthase complex is composed of 18 subunits which conform to $F_1$, the stalk, and $F_0$. It can be observed that $IF_1$ is present in the ATP synthase preparation ($F_0F_1I$). This was detected by Coomassie blue staining, but its presence was further confirmed by Western blot analysis using monospecific conventional antibodies (Fig. 2). It should also be noted that the ATP synthase complex isolated by this method contains two minor

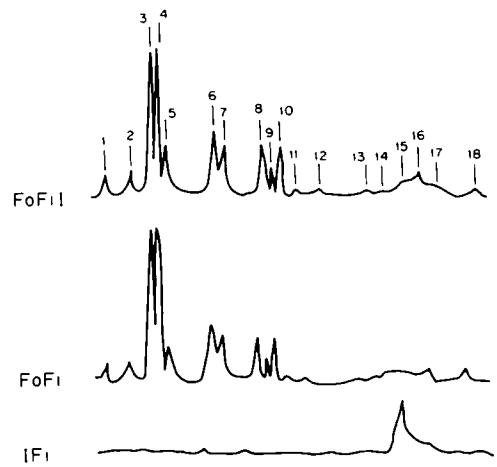



**Fig. 1.** Densitometric traces of $F_0F_1$-Inhibitor Protein, $F_0F_1$ complex, and $IF_1$ preparations. The gels were scanned in Ultroscan XL, LKB Bromma, Sweden. The proteins were separated by electrophoresis in the presence of sodium dodecyl sulfate on polyacrylamide gels as described in Materials and Methods.

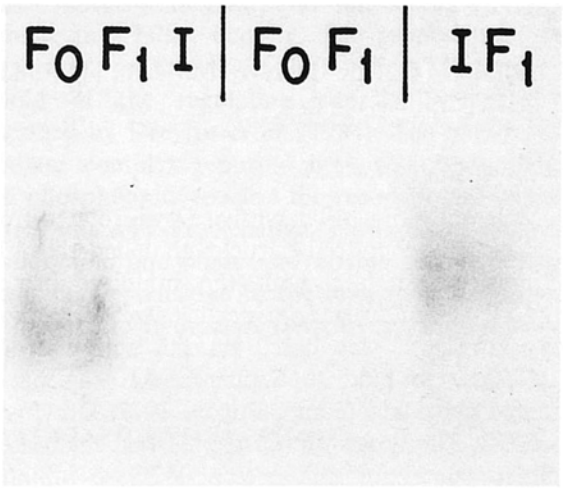



**Fig. 2.** Detection of $IF_1$ on the latent $F_0F_1$ ATP synthase by Western blot analysis. $F_0F_1I$ as well as $F_0F_1$ preparations were analyzed using polyclonal anti-$IF_1$ antibodies. The analysis was performed as described under Materials and Methods. The gel was loaded with 20 $\mu$g of either $F_0F_1I$ or $F_0F_1$ and 2 $\mu$g of $IF_1$.

**Table I.** Polypeptide Composition of the $F_0F_1$-Inhibitor Protein Complex[a]

| Band | Molecular weight | Subunit | | |
|---|---|---|---|---|
| | | $F_0$ | $F_1$ | Stalk |
| 1 | 85,090 | | | |
| 2 | 69,800 | | | |
| 3 | 54,567 | | $\alpha$ | |
| 4 | 50,998 | | $\beta$ | |
| 5 | 46,900 | | CK | |
| 6 | 34,700 | | | |
| 7 | 30,300 | | $\gamma$ | |
| 8 | 24,500 | "b" | | |
| 9 | 21,070 | | | OSCP |
| 10 | 19,700 | "d" | | |
| 11 | 18,500 | "a" | | |
| 12 | 15,000 | | $\delta$ | |
| 13 | 12,400 | | | $F_B$ |
| 14 | 11,900 | "A6L" | | |
| 15 | 10,800 | | | $F_6$ |
| 16 | 10,100 | | $IF_1$ | |
| 17 | 9,705 | "c" | | |
| 18 | 8,400 | | "$\epsilon$" | |

[a] The proteins were separated by electrophoresis in the presence of sodium dodecyl sulfate on polyacrylamide gel (10–23%) and stained with Coomasie Blue as described under Materials and Methods. CK corresponds to creatine kinase as reported by Lutter *et al.* (1993b).

components with molecular weights of 85,000 and 69,800 (see Table I). It also shows a band of molecular weight of 46,900 daltons that corresponds to creatine kinase as reported by Lutter *et al.* (1993b).

It is noteworthy that our preparations are essentially devoid of the adenine nucleotide carrier. This is of relevance since the carrier interferes with the reconstitution of the complex in isoelectric liposomes which was one of the aims of this work (*vide infra*).

*Kinetic Characterization*

In order to characterize the $F_0F_1$ ATP synthase in complex with $IF_1$, a steady-state kinetic analysis for MgATP was carried out studying $F_0F_1I$, and $F_0F_1$ (Fig. 3). For the $F_0F_1I$ Lineweaver–Burk plots showed that the complex had a $V_{max}$ of 1.3 $\mu$mol min$^{-1}$ mg$^{-1}$ and a $K_m$ for Mg-ATP of 148 $\mu$M (closed circles). The $F_0F_1$ ATP synthase devoid of $IF_1$ shows a $V_{max}$ of 8.3 $\mu$mol min$^{-1}$ mg$^{-1}$ and a $K_m$ for Mg-ATP of 142 $\mu$M (open circles). The ATPase activity of the latent $F_0F_1I$ ATP synthase displayed a broad pH optima with a maximum at pH 9.0 (Fig. 4). The specific activity at pH 9.0 was severalfold higher than at pH 6.8, suggesting an activation of ATP hydrolysis due to a pH-dependent dissociation of the inhibitor protein (*vide infra*). It has been previously reported that the latent state of the mitochondrial $F_1$ ATPase in complex with the inhibitor protein can be abolished by incubation at 60°C (Feinstein and Moudrianakis, 1984; Beltrán *et al.* 1988). Hence we subjected the $F_0F_1I$ ATP synthase, which exhibited low levels of ATPase activity, to a

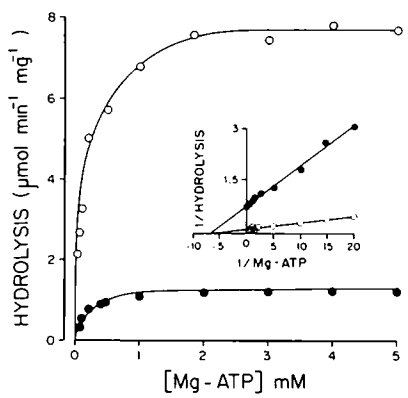



**Fig. 3.** Steady-state kinetic analysis for Mg-ATP of the latent and uncontrolled $F_0F_1$ ATP synthase. The $F_0F_1I$ complex shows a $V_{max}$ value of 1.28 $\mu$mol min$^{-1}$ mg$^{-1}$ and a $K_m$ for Mg-ATP of 148 $\mu$M (closed circles). On the other hand, the $F_0F_1$ ATP synthase devoid of $IF_1$ shows a $V_{max}$ of 8.33 $\mu$mol min$^{-1}$ mg$^{-1}$ and a $K_m$ for Mg-ATP of 142 $\mu$M (open circles). ATPase activity was assayed as indicated under Materials and Methods.

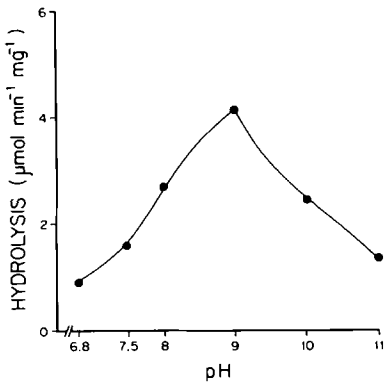
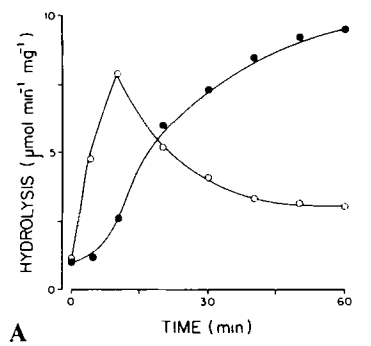



**Fig. 4.** Effect of pH on the ATPase activity of the $F_0F_1$ I complex. ATP hydrolysis was determined as described under Materials and Methods. Samples were incubated for 10 min at the pH indicated and ATP hydrolysis was started by the addition of 350 $\mu$l of reaction medium at 30 C.



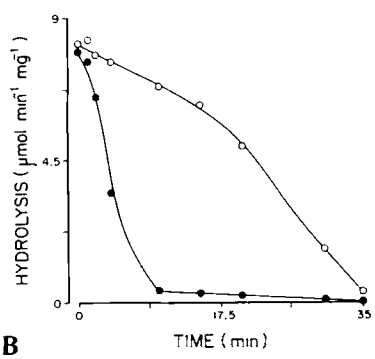




**Fig. 5.** (A) Effect of temperature (60 C) upon incubation of the $F_0F_1$ I complex, pH 6.8 (closed circles) and pH 9.0 (open circles). The ATP synthase (10–20 $\mu$g) was incubated at the temperature and pH indicated for 10 min. The samples were taken and assayed for ATP hydrolysis as described under Materials and Methods. (B) Effect of temperature (60 C) upon incubation of the $F_0F_1$ complex, pH 6.8 (closed circles) and pH 8.0 (open circles)

temperature of 60°C and a pH of 6.8. Under these conditions the ATPase activity gradually increased to a level 9.5 $\mu$mol min$^{-1}$ mg$^{-1}$ (Fig. 5A). However, if the incubation at 60°C was carried out at pH 9.0, ATP hydrolysis increased rapidly to a maximum value of 7.9 $\mu$mol min$^{-1}$ mg$^{-1}$; this was followed by a rapid drop in activity (Fig. 5A) that stabilized at about 3.0 $\mu$mol min$^{-1}$ mg$^{-1}$. This is higher than the rate at time zero.

Interestingly, when the $F_0F_1$ ATP synthase was subjected to high temperature at a pH of 8.0, a decrease in the rate of ATP hydrolysis was observed as a function of time (open circles) in Fig. 5B. Almost complete inhibition is attained after 35 min. The decrease in hydrolytic activity was very sharp when the incubation was carried out at pH 6.8; inactivation was almost complete in 10 min of exposure to high temperature (Fig. 5B, closed circles).

The latent ATPase activity of the $F_0F_1$-I ATP synthase could also be increased by incubation of the complex for 10 min with increasing concentrations of KCl. Complete activation was reached in the presence of 40 mM KCl (Fig. 6). It should be noted that the maximum activity reached was ca. 41% of the value obtained after 60 min at 60°C. The $F_0F_1$-ATP synthase complex devoid of $IF_1$ displayed a high hydrolytic activity insensitive to potassium chloride (inset, Fig. 6).

## Reconstitution of the ATP Synthase Complex in Liposomes and the ATP-Pi Exchange Reaction

The ATP synthase complex was reconstituted in liposomes (see Fig. 7) using the column filtration

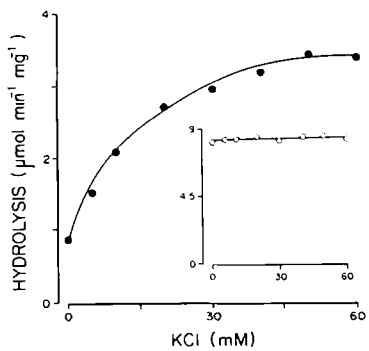



**Fig. 6.** Effect of increasing ionic strength on the ATPase activity of the $F_0F_1$ I (closed circles) and $F_0F_1$ complex (open circles). Latent or active $F_0F_1$ ATP synthase was incubated in 100 $\mu$l of buffer containing the indicated amounts of KCl. The hydrolysis of ATP was started by the addition of 250 $\mu$l of reaction medium at 30°C as described under Materials and Methods.

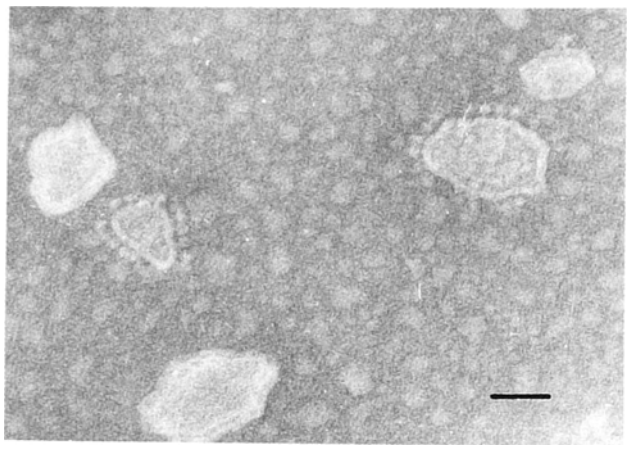


**Fig. 7.** Proteoliposomes of the reconstituted $F_0F_1I$ complex. Bar corresponds to 50 nm.

method described under Materials and Methods. The ATP-Pi exchange reaction was assayed in media of various compositions in order to determine the conditions for maximal exchange (see Table II). Table II shows the exchange activity using two different concentrations of inorganic phosphate with and without bovine serum albumin (BSA). It can be noted that the exchange reaction carried out by the proteoliposomes containing the $F_0F_1I$ complex responded to an increase in the concentration of phosphate, i.e., the activity increased from 2.5 with 2 mM $H_3PO_4$ to 6.4 nmol $min^{-1}$ $mg^{-1}$ with 15 mM phosphate. In the presence of BSA and low phosphate concentration, the exchange rate was 4.87, and with the higher phosphate concentration, the rate was 11.0 nmol $min^{-1}$ $mg^{-1}$. The ATP-Pi exchange reaction was inhibited by nearly 100% oligomycin or FCCP (0.8 $\mu$g and 1 $\mu$M final concentration, respectively). Interestingly, the ATP-Pi exchange activity of the $F_0F_1$ ATP synthase was significantly lower than of the latent $F_0F_1I$ regardless of the fact that the hydrolytic activity of the former preparation is higher than with a consequent higher capacity to build up an ATP-dependent electrochemical gradient. The exchange reaction catalyzed by $F_0F_1$ with or without $IF_1$ was almost completely inhibited by FCCP and oligomycin. This illustrates that the coupling between $F_1$ and the proton channel $F_0$ is well preserved after the column filtration treatment.

**Table II.** ATP-$^{32}$Pi Exchange Reaction of the $F_0F_1I$ and $F_0F_1$ Complex[a]

| Proteoliposomes | Additions | | ATP-Pi exchange (nmol $min^{-1}$ $mg^{-1}$) | | |
|---|---|---|---|---|---|
| | $H_3PO_4$ (mM) | BSA (mg/ml) | Control | +FCCP | +Oligomycin |
| $F_0F_1I$ | 2 | — | 2.5 | 0 | 0 |
| | 2 | 1 | 4.9 | 0 | 0 |
| | 15 | — | 6.4 | 0 | 0.1 |
| | 15 | 1 | 11.0 | 0 | 0.3 |
| $F_0F_1$ | 2 | — | 0.2 | 0 | 0 |
| | 2 | 1 | 0.7 | 0 | 0 |
| | 15 | — | 1.8 | 0 | 0 |
| | 15 | 1 | 4.1 | 0 | 0 |

[a] The ATP-Pi exchange reaction was assayed under various conditions in order to determine the optimal rate of the exchange reaction.

## DISCUSSION

The isolation technique used in the present study has allows us to obtain an ATP synthase that is functional and under control of the endogenous inhibitor protein. The complex is composed of 18 polypeptides. Its composition is similar to the preparations of Galante *et al.* (1979), Dreyfus *et al.* (1984), Laird *et al.* (1986), and Walker *et al.* (1991). It should be noted that the subunit composition of the ATPase complex varies slightly among the preparations reported in the literature; this could be related to the various methodologies that have been used. Of the abovementioned preparations only the one reported by Walker *et al.* (1991) contains endogenous $IF_1$. Therefore the true subunit composition of a functional $F_0F_1$ ATP synthase is still under debate. The preparation presented in this study contains the natural inhibitor protein $IF_1$ and is devoid of the adenine nucleotide carrier, in contrast to the preparation of Serrano *et al.* (1976). The presence of $IF_1$ as a component that copurifies with the complex was evidenced by Western blot analysis using specific polyclonal antibodies. It is important to note that an ATP synthase preparation such as the one presented in this study will be of great use in the study of the regulating mechanisms in the native enzyme.

The latent $F_0F_1I$ responds to heat activation in a manner similar to the latent and soluble $F_1I$ ATPase described by Feinstein and Moudrianakis (1984) and the controlled submitochondrial particles of Beltrán *et al.* (1988). The kinetic constants determined for the latent complex show a low rate of ATP hydrolysis and an affinity for ATP that is in accordance with the values reported for the reconstituted ATP synthase

complex studied by Stigall *et al.* (1978) and Laird *et al.* (1986) which are devoid of $IF_1$. It should be noted that the affinity for ATP was not modified by the presence or absence of $IF_1$. Furthermore, we performed determinations of the affinity for Mg-ATP under conditions of saturating amounts of $IF_1$ (data not shown); this did not modify the $K_m$ value for Mg-ATP. These results are of importance in the light of the fact that the $F_0F_1$ ATP synthase exhibits a higher hydrolytic activity and the ATP-Pi exchange activity is significantly lower than in the case of the latent enzyme. The structural integrity of the reconstituted complex was demonstrated by the nearly complete inhibition of the hydrolytic activity by oligomycin. On the other hand, at pH 9.0 the latent $F_0F_1I$ ATP synthase showed a specific activity which was severalfold higher than at pH 7.4, suggesting that at alkaline pH $IF_1$ is released from the enzyme. This is in accordance with the notion of Milgrom (1991) that an inactive form of $IF_1$ is formed at high pH, through the rapid conversion of an active acidic form to an inactive basic form of $IF_1$.

Many unsuccessful efforts to reconstitute the latent $F_0F_1I$ and activated ATP synthase complex $F_0F_1$ by dialysis were carried out during the development of this study; the enzyme was always inactivated after several hours of dialysis. This result may be related to the detergent used to solubilize the enzyme. We have previously reported that the ATP synthase complex solubilized from the membrane with lauryl dimethyl amino oxide (LDAO) (Dreyfus *et al.* 1984) could be reconstituted by dialysis. We have now developed a reconstitution technique in which a central step is the passage of the complex through centrifugation columns (Vázquez-Contreras *et al.*, unpublished results). The resulting ATP synthase complex exhibits hydrolytic activity and a high sensitivity to oligomycin, and can be readily incorporated into liposomes with or without $IF_1$. This allowed us to determine the extent of ATP-Pi exchange experiments in an integral $F_0F_1$ with and without endogenous $IF_1$ complex. These results are in contrast to those of Lutter *et al.* (1993a) who were not able to demonstrate either ATP-Pi exchange or ATP synthesis in a similar ATP synthase preparation. Both preparations catalyzed an ATP-Pi exchange reaction that was sensitive to FCCP and oligomycin. This reaction increased as the concentration of phosphate was increased, and bovine serum albumin increased phosphate uptake. The latter was probably due to a scavenger effect of detergent molecules that hinder the ATP-Pi exchange reaction. The latent $F_0F_1I$ ATP synthase has a higher specific exchange activity than the ATPase complex devoid of $IF_1$. This is particularly relevant, since the hydrolytic activity of $F_0F_1I$ ATP synthase is severalfold lower than that of complex without $IF_1$. Hence the controlled ATP synthase shows a tighter coupling between hydrolytic and synthetic reactions. We have compared the ATP-Pi exchange rates displayed by the $F_0F_1I$ and $F_0F_1$ ATP synthase preparations with those reported by Serrano *et al.* (1976), Stigall *et al.* (1978), and Dreyfus (1985). From previously reported data we cannot conclude if the absolute rate value for ATP-Pi exchange should be compared. A more meaningful term will be the ratio of ATP hydrolyzed to Pi uptake as this reflects the true value of energy conservation. This suggests that $IF_1$ would not only regulate catalysis by inhibiting ATP hydrolysis but would be acting as a real coupling devise that increases the efficiency of energy transduction. In fact, Guerrieri *et al.* (1987) have shown that $IF_1$ not only inhibits ATP hydrolysis, but also inhibits the proton conductivity of the complex. These authors suggest that the inhibitor protein could be involved in the gate of the $H^+$-ATPase.

There has been some discussion whether $IF_1$ is dissociated from the ATP synthase under energized conditions (see the review by Pedersen *et al.*, 1981; and Harris and Das, 1991). However, recent data showed convincingly that $IF_1$ remains in the ATP synthase under such conditions (López-Mediavilla *et al.*, 1993), which is in agreement with our previous observations (Dreyfus *et al.*, 1981).

The ATP synthase preparation presented in this study retains its controlled hydrolytic activity, due to the presence of endogenous $IF_1$. This provides the possibility of carrying out detailed studies on the regulation of the catalytic activity of the native latent ATP synthase complex and on the possible role that $IF_1$ might exert on energy conservation.

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