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Mitochondrial Adenosinetriphosphatase Inhibitor Protein: Reversible Interaction with Complex V (ATP Synthetase Complex)†

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ABSTRACT: Mitochondrial ATPase inhibitor protein (IF_1) reacts reversibly with complex V and inhibits up to 90% of its ATPase activity. Both the rate and extent of inhibition are pH and temperature dependent and increase as the pH is lowered from pH 8 to 6.7 (the lowest pH examined) or as the temperature is increased from 4 to 36 °C. Nucleotide triphosphates plus Mg^{2+} ions are required for inhibition of complex V ATPase activity by IF_1. In the presence of Mg^{2+} ions, the effectiveness order of nucleotides is ATP > ITP > GTP > UTP. Highly purified complex V, which requires added phospholipids for expressing ATPase and ATP–P_i exchange activities, cannot be inhibited by IF_1 plus ATP–Mg^{2+} unless phospholipids are also added. This indicates that the active state of the enzyme is necessary for the IF_1 effect to be manifested, because F_1–ATPase, which does not contain nor require phospholipids for catalyzing ATP hydrolysis, can be inhibited by IF_1 plus ATP–Mg^{2+} in the absence of added phospholipids. The IF_1-inhibited complex V, but not IF_1-inhibited F_1–ATPase, can be reactivated by incubation at pH >7.0 in the absence of ATP–Mg^{2+}. The reactivation rate is pH dependent and is influenced by temperature and enzyme concentration. Complex V preparations contain small and variable amounts of IF_1. This endogenous IF_1 behaves the same as added IF_1 with respect to conditions described above for inhibition and reactivation and can result in 25–50% inhibition in different complex V preparations. However, complex V lacking endogenous IF_1 can be reconstituted from F_0, F_1, oligomycin sensitivity conferring protein, and phospholipids. Inhibition of this reconstituted preparation in the presence of ATP–Mg^{2+} depends entirely on addition of IF_1. In general, the ATP–P_i exchange activity of complex V is more sensitive to the chemical inhibitors of F_1–ATPase than its ATPase activity. This is not so, however, for IF_1. Under conditions that IF_1 caused ~75% inhibition of ATPase activity of complex V, no more than 10% of the ATP–P_i exchange activity was inhibited.

The mitochondrial ATPase inhibitor protein (IF_1) is a low molecular weight, water-soluble, and heat-stable protein first discovered and isolated from bovine heart mitochondria by Pullman & Monroy (1963). Similar inhibitor proteins have also been isolated from rat liver (Chan & Barbour, 1976a; Cintron & Pedersen, 1979), *Candida utilis* (Satre et al., 1975), *Saccharomyces cerevisiae* (Ebner & Maier, 1977), chloroplasts (Nelson et al., 1972), and *Escherichia coli* (Nienvenhuis et al., 1974; Smith & Sternweis, 1977; Laget & Smith, 1979). The mitochondrial IF_1 has been extensively studied by various investigators (Horstman & Racker, 1970; Brooks & Senior, 1971; Ernster et al., 1973; Van de Stadt et al., 1974; Gómez-Puyou et al., 1977; Ferguson et al., 1977; Harris et al., 1979; for review, see Ernster et al., 1979). In chloroplasts and *E. coli*, the ATPase inhibitor protein constitutes the ϵ subunit of F_1–ATPase with a molecular weight of 15 000–16 000. However, in bovine heart mitochondria, IF_1 and the ϵ subunit of F_1 are distinct entities with molecular weights, respectively, of 10 500 and 5700–7500. It is generally believed

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that the beef heart IF_1 is released from F_1–ATPase by membrane energization via substrate oxidation (Asami et al., 1970; Van de Stadt et al., 1973; Ferguson et al., 1977), and it was originally thought that IF_1 is a unidirectional regulator of oxidative phosphorylation by inhibiting only the backflow of energy through ATP hydrolysis (Ernster et al., 1973). However, it is now believed that, in addition to inhibiting ATP hydrolysis, IF_1 also inhibits the initial rate of ATP synthesis (Gómez-Puyou et al., 1979; Harris et al., 1979) on the basis of the observation that in submitochondrial particles oxidative phosphorylation is preceded by a lag phase which appeared to be a function of the IF_1 content of the particles. Thus it was concluded that, before the onset of oxidative phosphorylation, F_1–ATPase undergoes a transition in response to membrane energization which results in the release of inhibition of IF_1. It should be pointed out, however, that in these studies an actual correlation was not made between the IF_1 content of the particles and the duration of the lag phase of oxidative phosphorylation. In fact, as in all the other reports in this area, the IF_1 content of submitochondrial particles was only *inferred* from the ATPase activity of the particle preparation used.

The present paper describes the reversible inhibition of the ATPase activity of complex V by IF_1, the conditions required for inhibition and release of ATPase activity, and the effect of IF_1 on the ATP–P_i exchange activity of complex V.

Methods and Materials

Complex V was prepared as before from mitochondria or submitochondrial particles (Stiggall et al., 1978, 1979). Purified, delipidated complex V was obtained by the procedure of Galante et al. (1979). IF_1 was purified essentially by the method of Horstman & Racker (1970) up to step 4, but the heat treatment (step 5) was omitted. At this stage, the preparation showed a slight contamination in the high molecular weight region of $NaDodSO_4$–polyacrylamide gels, which was removed as follows. The pellet from the last ammonium sulfate plus ethanol precipitation (step 4) was resuspended in a small volume of 5 mM Hepes[1]/NaOH, pH 7.5, and dialyzed overnight against the same buffer. The protein was adsorbed on a small column (1.4 × 6.5 cm) of DE-52 (Whatman) equilibrated in 5 mM Hepes/NaOH, pH 7.5. The column was washed with 30 mL of buffer and eluted first with 50 mL of 25 mM KCl and then with 50 mL of 50 mM KCl in 5 mM Hepes/NaOH, pH 7.5. IF_1 was eluted in the second step. The active fractions (2.3 mL each) were pooled, concentrated by ultrafiltration over a UM-2 membrane (AMICON), and stored at −70 °C. Urea-F_0 was prepared by resolution of complex V with urea/EDTA as described elsewhere (Galante et al., in preparation). F_1–ATPase and OSCP were purified by a combination of the procedures of Senior & Brooks (1970), MacLennan & Tzagoloff (1968), and Senior (1971), as detailed before (Galante et al., 1979). ATPase and ATP–$^{33}P_i$ exchange activities were measured as described by Stiggall et al. (1978), as was the preparation of sonicated soybean phospholipids. Purified soybean phospholipids were prepared as described by Ragan & Racker (1973). ATPase inhibitor activity was measured essentially by the procedure of Horstman & Racker (1970) in 10 mM TES/Tris or 10 mM potassium phosphate, both at pH 6.7, as specified in the figure legends. The order of additions to the preincubation mixture was buffer, complex V, IF_1, and ATP–Mg^{2+} at the concentrations indicated. Unless otherwise stated, the incubation was carried out at 30 °C for 15–20 min. Acrylamide gel electrophoresis in the presence of $NaDodSO_4$ was performed routinely by the procedure of Weber & Osborn (1976). Two-dimensional gel electrophoresis in the presence of $NaDodSO_4$ was performed as described by Ludwig & Capaldi (1979). The first dimension consisted of a 5-mm cylindrical Weber & Osborn type (1976) gel [12.5% acrylamide, 0.34% bis(acrylamide)]. The second dimension was on a 5-mm slab Swank & Munkres type (1971) gel [15% acrylamide, 0.5% bis(acrylamide)]. Protein was measured by the biuret method (Gornall et al., 1949) in the presence of 1% deoxycholate during the preparation of complex V or ATPase inhibitor and by the method of Lowry et al. (1951) or Bensadoun & Wenstein (1976) with the purified preparations.

Adenine nucleotides were obtained from Boehringer (Mannheim) or P-L Biochemicals; azolectin was from Associated Concentrates, Inc.; acrylamide and bis(acrylamide) were from Bio-Rad. [^{33}P]Phosphate was obtained from New England Nuclear and was recrystallized as described before (Stiggall et al., 1979). All other chemicals were reagent grade. Rutamycin was a gift from Eli Lilly & Co.

Results

The ATPase activity of complex V is reversibly inhibited up to 90% when the preparation is incubated with IF_1 under appropriate conditions. The active state of the enzyme, a suitable nucleotide triphosphate, and Mg^{2+} ions are required to achieve inhibition. pH values below 7.5 favor inhibition and above 7.0 favor reversal. Temperature, under other favorable conditions, increases the rate of inhibition as well as reversal. Before describing the details of the results, it is important, however, to explain how the experiments leading to inhibition and release of activity were conducted. For inhibition, the enzyme (i.e., complex V or F_1–ATPase) was incubated with IF_1 under the conditions specified. Then, samples were withdrawn at the indicated intervals and assayed for activity. For reversal of inhibition, the enzyme and other additives were incubated at 30 °C with predetermined amounts of IF_1 to achieve maximum inhibition. In this way, addition of excessive amounts of IF_1 was avoided. After maximum inhibition was reached, saturated ammonium sulfate was added to the level of 0.5 saturation, and the precipitated IF_1–enzyme complex was sedimented by centrifugation for 10 min at 105000*g*. The supernatant was discarded. The pellet was resuspended in buffered 0.25 M sucrose, incubated at the specified temperature, and sampled for activity assay at the intervals indicated.

Effects of pH and Temperature. The effect of pH on the inhibition of complex V ATPase activity by IF_1 and on the release of inhibition is shown in Figure 1 (A and B, respectively). It is seen that lowering of the pH of the incubation medium from pH 8.0 to 6.7 greatly increased the rate of inhibition, while the release of inhibition under the conditions described above was facilitated by increasing the pH of the suspension of the inhibited complex V from pH 6.7 to pH 8.0. Results similar to those of Figure 1A have been reported by others (Pullman & Monroy, 1963; Horstman & Racker, 1970; Gómez-Fernandez & Harris, 1978) with regard to the effect of pH on the IF_1 inhibition of soluble F_1–ATPase and AS particles (submitochondrial particles partially depleted of IF_1) (Racker & Horstman, 1967). However, the release of inhibition as a function of time and pH has not been described previously. The conditions required for achieving inhibition and reversal do not lend themselves to a satisfactory analysis

[1] Abbreviations used: IF_1, F_1–ATPase inhibitor protein; OSCP, oligomycin sensitivity conferring protein; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tes, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; Nbf-Cl, 4-chloro-7-nitrobenzofurazan; FSBA, 5′-(*p*-fluorosulfonylbenzoyl)adenosine.

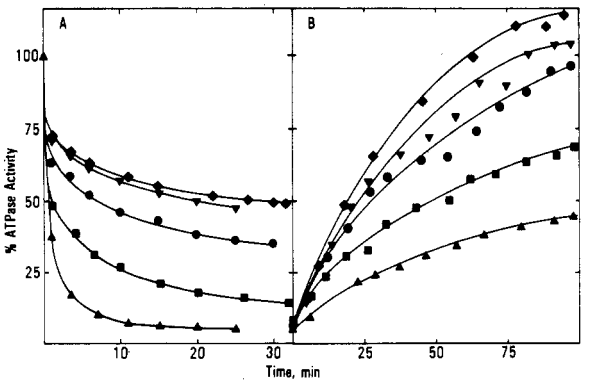

FIGURE 1: Effect of pH on (A) the inhibition of complex V ATPase activity by IF_1 and (B) the reversal of inhibition. (A) Complex V was incubated at a protein concentration of 1 mg/mL in 0.25 M sucrose containing 10 mM potassium phosphate at pH 6.7 (▲), 6.8 (■), 7.1 (●), 7.5 (▼), and 7.9 (♦) in the presence of 4 μg of IF_1 per unit of complex V ATPase activity and 1 mM ATP–Mg^{2+} at 30 °C. At the intervals shown, aliquots were withdrawn and assayed for ATPase activity as described under Methods and Materials. The specific activity of complex V in the absence of added IF_1 was 7 μmol min^{-1} (mg of protein)$^{-1}$. (B) Complex V at 1.2 mg of protein/mL was incubated for 20 min with 3–4 μg of IF_1 per unit of complex V ATPase activity (specific activity 9 μmol min^{-1} mg^{-1}) as above. The mixture was brought to 0.5 saturation with saturated, neutralized ammonium sulfate solution, and centrifuged for 10 min at 105000g. The pellet was resuspended at a protein concentration of 6–8 mg/mL in 0.25 M sucrose containing 50 mM potassium phosphate, pH 6.7 (▲), 6.8 (■), 7.0 (●), 7.5 (▼), and 8.0 (♦). ATPase activity was assayed as above.

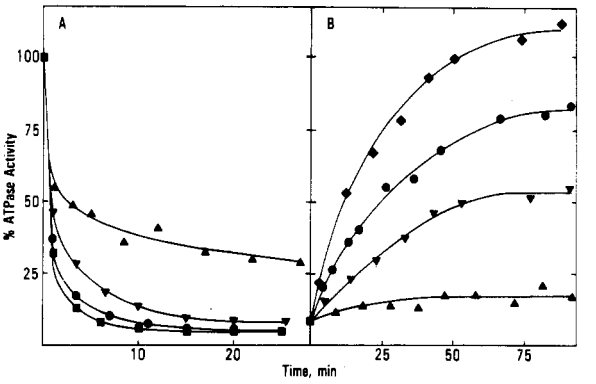

FIGURE 2: Effect of temperature on (A) the inhibition of complex V ATPase activity by IF_1 and (B) the reversal of inhibition. (A) Complex V at a protein concentration of 1 mg/mL was incubated as in Figure 1A at pH 6.7 with 7 μg of IF_1 per unit of complex V ATPase activity (specific activity 8.5 μmol min^{-1} mg^{-1}) at 0 (▲), 20 (▼), 30 (●), and 36 °C (■). (B) IF_1-inhibited complex V was prepared as in Figure 1B and incubated at 8 mg/mL in 0.25 M sucrose containing 50 mM potassium phosphate, pH 8.0, at the temperatures indicated above. ATPase activity was assayed as in Figure 1.

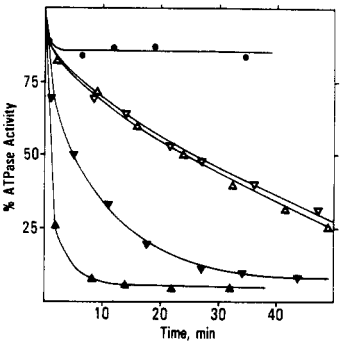

FIGURE 3: Effects of ATP and ADP ± Mg^{2+} ions on the inhibition of complex V ATPase activity by IF_1. Complex V was incubated as in Figure 2A with 3.5 μg of IF_1 per unit of ATPase activity (specific activity 8 μmol min^{-1} mg^{-1}) in the absence (●) or presence of 2 mM ADP (▽), 2 mM ATP (△), 2 mM ADP–Mg^{2+} (▼), or 2 mM ATP–Mg^{2+} (▲). ATPase activity was assayed as in Figure 1.

of the initial rates of inhibition and reversal as shown in Figure 1. Nevertheless, plots of the logarithm of the extent of inhibition or release of activity after a given short period of incubation (see Figure 1) vs. pH clearly indicated slope changes at pH 6.8–7.0, suggesting involvement of protein residues with near-neutral pK values.

F_1–ATPase was also inhibited by IF_1 under the same conditions used in Figure 1A for inhibition of the ATPase activity of complex V. However, we were not able to reverse the inhibition of F_1–ATPase by incubation at pH 7.5–8.5 and elevated temperatures, even in the presence of added phospholipids. Another difference observed was that complex V required approximately 0.45 mol of IF_1 per mol for maximum inhibition while F_1–ATPase required nearly 0.8 mol of IF_1 per mol. The latter result is in agreement with the calculations of Gômez-Fernandez & Harris (1978) for AS particles and the values deduced by Klein et al. (1980) from labeled IF_1 binding to F_1–ATPase. The lower titer for complex V might be related to the fact that complex V preparations contain variable levels of endogenous IF_1 which, under appropriate conditions, can result up to 50% inhibition of ATPase activity (see below).

The effect of temperature on inhibition and reversal is shown in Figure 2 (A and B, respectively). The data of Figure 2A agree in general with the results of Gômez-Fernandez & Harris (1978) for inhibition of the ATPase activity of AS particles. These authors have calculated the standard enthalpy and entropy of IF_1 binding to AS particles to be in the range of about +9 to +11 kcal/mol and +0.06 to +0.07 eu, respectively. As seen in Figure 2B, the effect of temperature on the reversal of inhibition of complex V is much more pronounced than on inhibition (Figure 2A). However, whether the reversal of inhibition results from the release or displacement of the bound IF_1 remains to be seen. At any rate, the activation enthalpy and entropy calculated from the data of Figure 2B for the release of inhibition at pH 6.7 were $\Delta H^{\ddagger}$ = +14.3 kcal/mol and $\Delta S^{\ddagger}$ = −0.03 eu.

Requirement for Substrates. As indicated by others for membrane-bound and soluble F_1–ATPase (Horstman & Racker, 1970; Gômez-Fernandez & Harris, 1978), inhibition of the ATPase activity of complex V by IF_1 requires the presence of a hydrolyzable nucleoside triphosphate and Mg^{2+} ions in the incubation medium. Figure 3 shows the effects of ATP–Mg^{2+}, ADP–Mg^{2+}, ATP, and ADP vs. no nucleotide addition. The inhibition by IF_1 in the presence of ADP–Mg^{2+} is probably related to the fact that preparations of complex V contain adenylate kinase activity (see Table I), and the slow inhibition by ATP or ADP in the absence of added Mg^{2+} ions might be referable to the presence of Mg^{2+} ions in the preparation. Indeed, analyses of complex V purified on an Agarose column (Galante et al., 1979) showed the presence of appreciable amounts of Mg^{2+} in the preparation, of which about 0.5 mol of Mg^{2+} per mol of complex was very tightly bound and could not be removed by prolonged dialysis (Dr. Alan Senior, private communication). Figure 4 shows the effects of ITP, GTP, and UTP as compared to ATP, all in the presence of Mg^{2+} ions, and Table I compares the amount of P_i released from the above substrates after 10 min of incubation with complex V under the same conditions used for IF_1 inhibition in Figure 3 and 4. These results suggest a correlation between enzyme turnover and manifestation of IF_1 effect and agree with the previous conclusions of Gômez-Fernandez & Harris (1978). However, it should be added that the V_{max} values for complex V catalyzed hydrolysis of ATP, GTP, and

Table I: Hydrolytic Activity of Complex V with Different Nucleotides as Substrates[a]

nucleotide	specific act.
ATP-Mg^{2+}	7400
ITP-Mg^{2+}	3550
GTP-Mg^{2+}	2900
UTP-Mg^{2+}	300
ADP-Mg^{2+}	85
ATP	50
ADP	4

[a] Complex V (3.5 μg) was incubated in 0.25 M sucrose containing 50 mM Tris-acetate and 25 mM potassium acetate (pH 7.5) at 30 °C for 10 min in the presence of 2 mM nucleotide ± Mg^{2+} as indicated. The reactions were stopped by addition of 4% trichloroacetic acid. Denatured protein was removed by centrifugation, and phosphate was determined in the supernatant according to Sumner (1944). All the reaction rates, except in the presence of ATP-Mg^{2+} as substrate, were linear with time, at least up to 20 min. Specific activity is given as nmol min^{-1} (mg of protein)$^{-1}$.

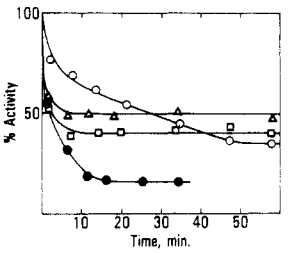

FIGURE 4: Effects of GTP (△), ITP (□), and UTP (○) as compared to ATP (●) on the inhibition of complex V ATPase activity by IF_1. Complex V at 1.2 mg of protein/mL of 0.25 M sucrose containing 10 mM Tes/Tris, pH 6.8, was incubated with 3 μg of IF_1 per unit of complex V ATPase activity (specific activity 9 μmol min^{-1} mg^{-1}) in the presence of 2 mM NTP-Mg^{2+} at 30 °C as indicated. ATPase activity was assayed as in Figure 1.

ITP are very similar, i.e., 8–10, 6, and 4 μmol min^{-1} mg^{-1}, respectively, while the K_m values for ITP and GTP are 40- to 100-fold higher than the K_m for ATP (Stiggall et al., 1978). We therefore checked the effect of saturating levels ($5K_m$) of ATP, GTP, and ITP plus Mg^{2+} ions on IF_1 inhibition of complex V ATPase activity. The degrees of inhibition observed after 20 min of incubation were 86% for ATP, 88% for ITP, and 38% for GTP.

Requirement for Active Enzyme. According to Gómez-Fernandez & Harris (1978), enzyme turnover (200 times or more) is necessary for the ATPase to combine with IF_1. This conclusion is in apparent agreement with the data of Figure 4 and Table I. However, a more stringent test that the functional state of the enzyme is required for activity inhibition by IF_1 was performed with the use of dilipidated complex V. These preparations of complex V, purified on Agarose columns in the presence of cholate and ammonium sulfate, contain very low levels of phospholipids (3–4 μg of phospholipid phosphorus per mg of protein) and required added phospholipids in the assay mixture in order to catalyze ATP hydrolysis and ATP-P_i exchange (Galante et al., 1979). As seen in Figure 5, incubation of delipidated complex V with various amounts of IF_1 in the presence of ATP-Mg^{2+}, but in the absence of added phospholipids, resulted in little or no inhibition, while in the presence of added phospholipids considerable inhibition was obtained at the lowest level of IF_1 used. It should be pointed out that in these experiments added phospholipid was always present at the constant saturating level of 20 mM in the ATPase assay mixture, since otherwise the delipidated enzyme would not exhibit any activity. However, the presence of phospholipids in the assay mixture was not a complicating factor, because (a) the ATPase assay was performed spectrophotometrically and the initial rates were measured, and (b) the pH of the assay medium was not expected to promote rapid enzyme–IF_1 interaction. On the other hand, in order to study the correlation between ATPase activity and inhibition by IF_1, the experiments were performed differently. In this case, increasing amounts of phospholipids were added to two sets of incubation mixtures, (A) containing only complex V and (B) containing complex V, ATP-Mg^{2+}, and IF_1. Then after 20 min of incubation at 30 °C, the two sets of incubation mixtures A and B were tested for ATPase activity as follows. The ATPase activity of set A was measured in the absence of added phospholipids to the assay medium to show the degree of activity elicited by the amount of phospholipid added to the preincubation mixtures. Set B was assayed in the presence of optimal amounts of added phospholipids in the assay mixture so that the full extent of ATPase activity not inhibited by IF_1 could be realized. The results given in Figure 6 show that as ATPase activity increased as a function of added phospholipids in the incubation mixture A, so did the inhibition of this activity by IF_1 in incubation mixture B. An obvious question posed by the data of Figures 5 and 6 is whether phospholipids are also required for inhibition of ATPase activity by IF_1. However, this does not appear to be the case, as is indicated by the fact that soluble F_1-ATPase neither contains nor requires phospholipids for activity. Yet its activity is inhibited upon incubation with IF_1 and ATP-Mg^{2+} in the absence of added phospholipids (Horstman & Racker, 1970; Senior, 1979; Penefsky, 1979).

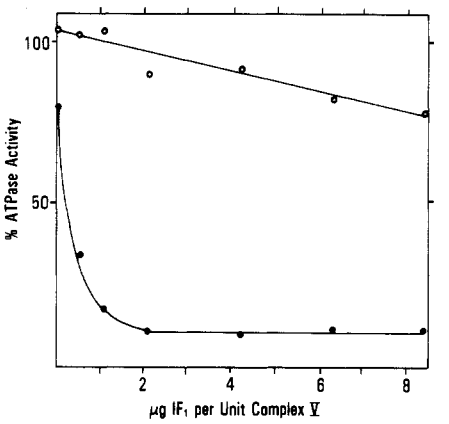

FIGURE 5: Inhibition of the ATPase activity of purified, delipidated complex V by IF_1. Purified complex V was incubated at a protein concentration of 1 mg/mL in 0.25 M sucrose containing 10 mM potassium phosphate, pH 6.7, at 0 °C in the absence (○) or presence (●) of 2.7 μmol of sonicated soybean phospholipids/mg of protein. After 10 min, the indicated amounts of IF_1 plus 2 mM ATP-Mg^{2+} were added, and the mixtures were incubated at 30 °C for 20 min. ATPase activity was measured in an assay mixture containing 20 mM phospholipids. 100% ATPase activity was 10 μmol min^{-1} (mg of protein)$^{-1}$.

Effect of IF_1 on the ATP-P_i Exchange Activity of Complex V. In general, the known chemical inhibitors of membrane-bound ATPase (e.g., rutamycin, venturicidin, dicyclohexylcarbodiimide, trialkyltin compounds, phenylglyoxal, butanedione, ferrous bathophenanthroline, mercurials, etc.) exhibit comparable or greater inhibitory effects on ATP-P_i exchange activity (Stiggall et al., 1978; Frigeri et al., 1977). IF_1 appears to be an exception, however, because as seen in Figure 7 it inhibits ATP hydrolysis much more than ATP-P_i exchange. First of all, as seen in Figure 7, incubation of complex V with ATP-Mg^{2+} at pH 6.7 in the absence of added IF_1 resulted in about 50% inhibition of ATPase activity by the endogenous IF_1 of complex V (see below). Under the same conditions, ATP-P_i exchange activity was, if anything, slightly activated.

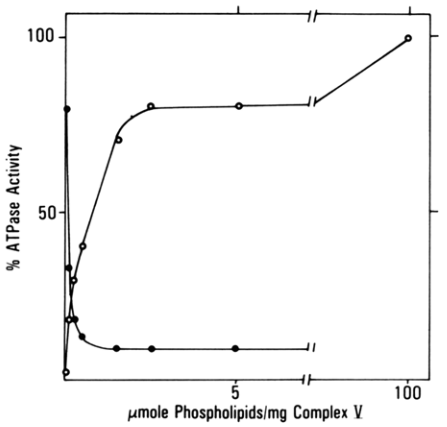

FIGURE 6: Effect of phospholipids on the ATPase activity of purified, delipidated complex V in the absence and presence of IF_1. Purified complex V was incubated in two sets of tubes A and B at a protein concentration of 1 mg/mL in 0.25 M sucrose containing 10 mM potassium phosphate, pH 6.7, at 0 °C in the presence of the indicated amounts of soybean phospholipids in each tube of each set. After 10 min, 7 μg of IF_1 per unit of complex V ATPase activity plus 2 mM ATP–Mg^{2+} were added to tubes of set B and a comparable volume of buffer was added to tubes of set A. The mixtures were then incubated for an additional 20 min at 30 °C. Mixtures A (○) were assayed for ATPase activity in the *absence* of added phospholipids to the assay medium, and mixtures B (●) were assayed as in Figure 5 in the presence of optimal amounts of added phospholipids in the assay medium. 100% ATPase activity was 10 μmol min^{-1} (mg of protein)$^{-1}$.

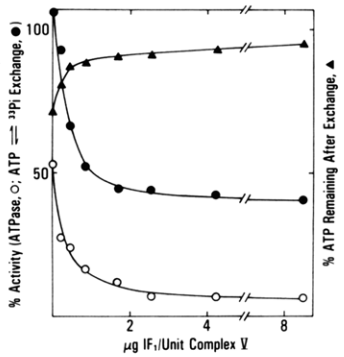

FIGURE 7: Effect of IF_1 on ATPase and ATP–$^{33}P_i$ exchange activities of complex V. Complex V was incubated at a protein concentration of 1 mg/mL in 0.25 M sucrose containing 10 mM Tes/Tris, pH 6.7, in the presence of the indicated amounts of IF_1 plus 2 mM ATP–Mg^{2+} at 30 °C for 30 min; 100 μg was withdrawn and assayed for ATP–$^{33}P_i$ exchange (●) as described under Methods and Materials, while 20 μg was incubated under the same conditions of the exchange reaction for determination of ATPase activity at the end of the exchange (○). Triangles (▲) show the amount of ATP remaining at the end of the exchange reaction; 100% activities were 9.0 μmol of ATP hydrolyzed min^{-1} mg^{-1} and 180 nmol of $^{33}P_i$ exchanged min^{-1} mg^{-1}. ATP–P_i exchange was assayed in the absence of added phospholipids, and the rates were corrected for ATP hydrolysis during the exchange reaction time.

In the presence of added IF_1, both activities were then inhibited in parallel, with ATPase leveling off at about 93% inhibition and ATP–P_i exchange at about 60% inhibition. The triangles in Figure 7 show the percent of ATP remaining in the ATP–P_i exchange assay mixture at termination of the reaction after 10 min. They indicate that ATP hydrolysis in the ATP–P_i exchange assay mixture was inhibited by added IF_1 essentially in the same manner as it was in the direct ATPase assay system.

Endogenous IF_1 of Complex V Preparations. As seen in Figures 5 and 7, incubation of complex V with ATP–Mg^{2+} in the absence of added IF_1 resulted in partial inhibition of ATPase activity. The degree of inhibition varies with different

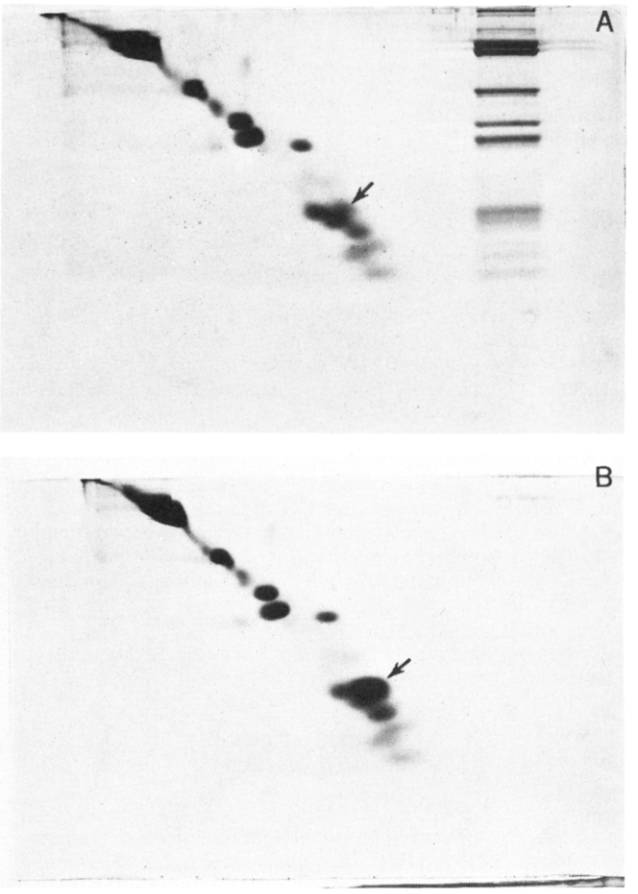

FIGURE 8: Two-dimensional gel electrophoresis pattern of complex V in the absence (A) and presence (B) of added IF_1. Complex V (A, 140 μg without added IF_1; B, 120 μg plus 18 μg of IF_1) was treated with $NaDodSO_4$ and electrophoresed on a cylindrical gel (12.5% acrylamide), using the Weber–Osborn (1976) system. The cylindrical gel was cemented onto a slab gel (15% acrylamide) and electrophoresed on a second dimension, using the Swank–Munkres (1971) system as described by Ludwig & Capaldi (1979). The lateral well of (A) contained 45 μg of $NaDodSO_4$-treated complex V. The gels were stained with Coomassie blue and destained according to Fairbanks et al. (1971). The arrows in the figures show the position of the endogenous IF_1 of complex V (A) and the same enriched with added IF_1 (B).

preparations of complex V between 25 and 50%. This inhibition appeared to be due to the presence of variable amounts of IF_1 in complex V preparations. Figure 8A shows a two-dimensional $NaDodSO_4$ gel electrophoresis of complex V in which the polypeptide identified as IF_1 has been marked with an arrow, and Figure 8B is the gel electrophoresis pattern of the same preparation of complex V in the presence of added IF_1, showing increased stain intensity of the IF_1 spot. The endogenous IF_1 seemed to behave the same as added IF_1 with respect to the ATPase activity of the enzyme since, as shown in Figure 9, it responded similarly to pH for causing inhibition (Figure 9A) and reversal (Figure 9B) and exhibited a similar requirement for ATP–Mg^{2+} (Figure 9A). This endogenous IF_1 could be removed when complex V was resolved with 4 M urea in the presence of dithiothreitol and EDTA (manuscript in preparation). The resulting IF_1-free F_0 was recombined with highly purified F_1 and OSCP in the presence of phospholipids to reconstitute an ATPase complex with rutamycin-sensitive ATPase and ATP–P_i exchange activities. Two-dimensional gel analysis of the reconstituted preparation showed the absence of detectable amounts of IF_1. As seen in Table II, incubation of the reconstituted preparation with ATP–Mg^{2+} in the absence of added IF_1 resulted in little or

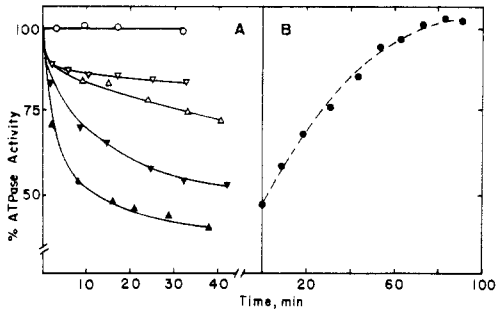

FIGURE 9: Reversible inhibition of complex V ATPase activity by endogenous IF_1. (A) Complex V was incubated at a protein concentration of 1 mg/mL in 0.25 M sucrose containing 10 mM potassium phosphate, pH 6.7, at 30 °C with no addition (○) or in the presence of 2 mM ADP (▽), 2 mM ATP (△), 2 mM ADP–Mg^{2+} (▼), or 2 mM ATP-Mg^{2+} (▲). At the times indicated, aliquots were withdrawn and assayed for ATPase activity. After 40 min, the mixture containing 2 mM ATP–Mg^{2+} (▲) was brought to 0.5 saturation with saturated, neutralized ammonium sulfate and centrifuged at 105000*g* for 10 min. The pellet was resuspended at a protein concentration of 6 mg/mL in 0.25 M sucrose containing 50 mM potassium phosphate, pH 8.0, incubated at 30 °C, and assayed for ATPase activity as shown in (B); 100% specific activity was 8 μmol min^{-1} (mg of protein)$^{-1}$.

Table II: ATPase Activity of Reconstituted Complex V in the Absence and Presence of Added IF_1[a]

additions	specific act.
none	15.8
ATP-Mg^{2+}	14.5
IF_1 + ATP-Mg^{2+}	4.3

[a] F_0 (obtained from complex V by urea/EDTA resolution) was sonicated at 0 °C for 30 s at a protein concentration of 1.6 mg/mL in the presence of 10 μmol of sonicated, purified soybean phospholipids per mg of F_0 protein, 0.4% potassium cholate, 0.25 M sucrose, and 50 mM Tris-acetate (pH 7.5); 0.25 mg of F_1 and 75 μg of OSCP per mg of F_0 (i.e., 3 mol of OSCP per mol of F_1) were added, and the mixture was incubated at 30 °C for 30 min. The mixture was further incubated at 0 °C for 1 h to inactivate free F_1. At this point the ATPase activity was 15.6 μmol min^{-1} (mg of F_0)$^{-1}$ (92% rutamycin sensitive). The pH was lowered to 6.5–7.0 with 1 M acetic acid, IF_1 (3 μg/unit of ATPase activity) and/or 2 mM ATP-Mg^{2+} were/was added where indicated, and the incubation continued at 30 °C for 30 min before assaying again for ATPase activity. Specific activity is expressed as μmol min^{-1} (mg of F_0)$^{-1}$.

no inhibition of ATPase activity while incubation in the presence of added IF_1 caused inhibition.

In spite of the above results, the data of Figure 7 seem to suggest a difference in the effects of endogenous and added IF_1 on ATP–P_i exchange activity. The former caused no inhibition of ATP–P_i exchange while inhibiting ATPase activity by 50% (Figure 7); the latter caused at all levels a somewhat greater percent inhibition of ATP–P_i exchange than of ATPase activity. Yet ATPase activity could be inhibited by as much as 93%, while ATP–P_i exchange activity could not be inhibited more than about 60% even in the presence of very high levels of added IF_1. These results seem to suggest that added IF_1 may be different from the endogenous IF_1 in its structure and/or mode of interaction. It is also possible that ATP hydrolysis and ATP–P_i exchange are inhibited by IF_1 in different manners and/or at different sites.

Discussion

It has been shown in the present communication that IF_1 binds to complex V and results in inhibition of ATPase activity. In agreement with the previous findings of others on IF_1 binding to F_1 or submitochondrial particles (Horstman & Racker, 1970; Gómez-Fernandez & Harris, 1978), the inhibition is favored by pH <7.5 and increase of temperature and requires the presence of a hydrolyzable nucleoside triphosphate plus Mg^{2+} ions. In addition, our studies with delipidated complex V, which requires added phospholipids for activity, have indicated that inhibition by IF_1 also requires an active ATPase. Thus, delipidated complex V under otherwise optimal conditions could not be inhibited by IF_1 in the absence of added phospholipids while graded amounts of added phospholipids elicited comparable degrees of ATPase activity and IF_1 response in parallel experiments. That phospholipids do not appear to be required for IF_1 inhibition is evident from the fact that lipid-free F_1–ATPase preparations, which do not require lipids for activity, were inhibited by the same preparation of IF_1 which did not inhibit delipidated, inactive complex V. Gómez-Fernandez & Harris (1978) have suggested that a large number (about 200 or more) of enzyme turnovers are necessary for the ATPase to combine with IF_1. Whatever the precise meaning of a requirement for multiple turnovers, it is clear from our results that (a) the active state of the enzyme is required and (b) there appears to be a rough correlation between hydrolytic activity of the enzyme in the presence of a given substrate and the extent (possibly also the rate) of inhibition by IF_1.

It has been shown by Van de Stadt et al. (1974) that uncoupler-stimulated ATPase activity of submitochondrial particles increases by preincubation of the particles with an oxidizable substrate. They have explained that the increase in ATPase activity results from dissociation of IF_1 from membrane-bound F_1 induced by membrane energization. However, this interpretation did not rest upon actual measurement of IF_1 release. Subsequently, Bruni et al. (1977) showed that incubation of submitochondrial particles in the presence of an oxidizable substrate (e.g., succinate) followed by centrifugation resulted in the release of IF_1 which was recovered from the supernatant. However, estimation of IF_1 bound to the sedimented particles showed that they contained roughly as much IF_1 as the control particles not energized by an oxidizable substrate. These results would suggest that the amount of IF_1 released into the medium, apparently as a result of membrane energization, might not be a very significant portion of the total IF_1 content of the particles. On the other hand, it should be considered that all the above estimations of the IF_1 content of particles were indirect and inferred from measurement of ATPase activity which, as indicated by our results, can be subject to error. Thus, as shown above, complex V preparations can contain enough IF_1 to cause up to 50% inhibition of ATPase activity. However, this inhibition is not expressed unless the preparation is incubated at pH <7.5 in the presence of ATP–Mg^{2+} and can be reversed at pH >7.0 in the absence of ATP–Mg^{2+}.

Although the conclusion regarding IF_1 release by membrane energization needs to be reexamined by direct estimation of particle-bound IF_1, the fact that membrane energization results in increased ATPase activity is suggestive of regulation and is in agreement with out results regarding modulation of IF_1 effect by pH. Thus, it is conceivable that in submitochondrial particles the mechanism of $\Delta\mu_{H^+}$-induced release of ATPase inhibition as observed by the above workers is in fact due mainly to ionization of appropriate protein residues resulting either from F_1 conformation change or from a pH change in the vicinity of F_1–IF_1. That under otherwise appropriate conditions such a pH change need not be very large or far removed from neutrality to effect inhibition or reversal is clear from the results shown in Figure 1A,B.

Regarding reversal of IF_1 inhibition, the observation that the release of inhibition is favored at pH >7.0 is in agreement

with the matrix pH conditions when the mitochondria are energized and poised for ATP synthesis. The rate of inhibition release under the conditions used in this report are clearly slow. However, we have observed recently that replacement of phosphate with bicarbonate buffer at the same pH and ionic strength augments the rate of reversal considerably. Therefore, specific ions and other factors present in the mitochondrial matrix might have considerable effects on the reversal of IF_1 inhibition and would have to be investigated. In this connection, Gómez-Fernandez & Harris (1978) have concluded that the interaction of IF_1 with F_1 is mainly hydrophobic, while Horstman & Racker (1970) and Van de Stadt & Van Dam (1974) have shown that high ionic strength favors "dissociation" of IF_1. Whether or not hydrophobic interactions play a major role in F_1–IF_1 interaction, the salt effects and our pH data clearly point to the essentiality of ionizable groups for IF_1 inhibition and reversal.

As stated above, the conditions that bring about the release of IF_1 inhibition of complex V did not reverse the inhibition of soluble F_1. This may be related to the fact that soluble F_1 is known to be structurally and functionally different from its membrane-bound form. In addition, Chan & Barbour (1976b) have suggested that rat liver IF_1 interacts at multiple sites, including the membrane portion of the ATPase complex. Klein et al. (1980) have shown recently that IF_1 binds to the β subunit of soluble F_1. Whether in view of the above observations this is also true for the membrane-bound F_1 remains to be determined. As will be shown elsewhere (Y. M. Galante et al., unpublished data), pretreatment of complex V with IF_1 under conditions that resulted in activity inhibition did not prevent interaction and independent inhibition of complex V by Nbf-Cl, FSBA, ferrous bathophenanthroline, efrapeptin, aurovertin, phenylglyoxal, and octylguanidine. Thus, on the one hand, the β subunit is considered to bear the ATPase active site and, according to Klein et al., (1980) to bind IF_1. On the other hand, the above inhibitors, which are considered to bind at or near the ATPase active site, do not seem to bind at the same locus as IF_1. We do not think that these results are contradictory, but we feel that our studies with the above chemical inhibitors indicate that inhibition by IF_1 does not appear to be simply a masking of the ATPase active site. This view agrees with the findings that (a) in chloroplasts and *E. coli* the ϵ subunit, which is considered to be the counterpart of beef heart IF_1, appears to be in close association with the γ subunit rather than the β, and (b) in *E. coli* the ϵ subunit is involved in the binding of F_1 to the membrane (Nelson et al., 1972; Deters et al., 1975; Nelson, 1976; Baird & Hammes, 1976; Holowka & Hammes, 1977; Smith & Sternweis, 1977; Larson & Smith, 1977; Sternweis, 1978).

Finally, we wish to propose that IF_1 interaction with membrane-bound F_1 involves two distinct stages. It is clear from the previous work of others that IF_1 can be extracted and recombined with ATPase and that membrane energization does result in IF_1 release, at least partially. On the other hand, it is clear also from our results on the endogenous IF_1 of complex V that (a) the inhibitor protein can be very tightly bound to the particles and resist dislodgment during complex V purification, which involves treatment with detergents and high salt concentration at pH 8.0 and filtration through Sephadex and agarose in the presence of cholate and ammonium sulfate, and (b) it can remain particle bound in a non-inhibiting state but capable of reversible inhibition under appropriate conditions. These results suggest, therefore, a two-stage interaction between IF_1 and membrane-bound F_1, as shown in eq 1, in which $F_1 \cdot IF_1$ represents IF_1 binding

$$F_1 + IF_1 \rightleftharpoons F_1 \cdot IF_1 \underset{\text{pH} > 7.0}{\overset{\text{ATP-Mg}^{2+},\ \text{pH} < 7.5}{\rightleftharpoons}} F_1\text{–}IF_1 \qquad (1)$$

without causing activity inhibition, while F_1–IF_1 indicates a change in the mode of IF_1 binding which results in inhibition. It is reasonable to assume that in the mitochondrial matrix regulation of ATPase function involves only changes in the state of IF_1 binding, as shown by the reaction $F_1 \cdot IF_1 \rightleftharpoons F_1$–$IF_1$ rather than the release and rebinding of IF_1 as well. Indeed, whether the interaction of released IF_1 with the ATPase complex depicts a physiological phenomenon should be carefully examined. As was discussed earlier in connection with Figure 7, endogenous and added IF_1 appear to act very differently with respect to inhibition of ATP–P_i exchange. Endogenous IF_1, while inhibiting ATPase activity by 50%, resulted in no inhibition of ATP–P_i exchange; added IF_1 brought the inhibition of ATPase activity to 93% but did not cause more than 60% inhibition of ATP–P_i exchange activity. The low level of inhibition of ATP–P_i exchange activity by IF_1 may be related to the observation of Harris et al. [see Figures 1 and 2 in Harris et al. (1979)] that ATP synthesis under steady-state conditions is not inhibited by IF_1.

Acknowledgments

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Regulation of Procollagen Messenger Ribonucleic Acid Levels in Rous Sarcoma Virus Transformed Chick Embryo Fibroblasts†

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ABSTRACT: Using cloned cDNAs for pro-α1 and pro-α2 collagen messenger ribonucleic acid (mRNA), we have investigated the regulation of collagen mRNA levels in Rous sarcoma virus (RSV) transformed chick embryo fibroblasts (CEF). We find that both pro-α1 and pro-α2 mRNA levels are decreased ~10-fold in CEF transformed by either the Bryan high-titer strain or the Schmidt–Ruppin strain of RSV. Using temperature-sensitive mutants in the transforming gene *src*, we also investigated the rate of change in the levels of the two mRNA species. We employed mutants of both the Bryan high-titer strain (BHTa) and the Schmidt–Ruppin strain (ts68). With both mutants the results were similar. Upon shift from the permissive temperature (35 °C) to the nonpermissive temperature (41 °C), collagen mRNA synthesis did not increase until more than 5 h had passed, suggesting the action of *src* on collagen gene expression is indirect. Upon shift from 41 to 35 °C, collagen mRNA levels fell with a half-life of 10 h. Whether this fall reflects the half-life of procollagen mRNA or an effect of *src* on procollagen RNA stability is unclear. Both pro-α1 and pro-α2 mRNA levels were coordinately controlled.

Fibroblasts which are transformed by ribonucleic acid (RNA)[1] tumor viruses show a variety of altered properties (Hanafusa, 1977; Pastan & Willingham, 1978). One major alteration is the decrease in collagen gene expression which follows transformation of chick embryo fibroblasts (CEF)[1] by Rous sarcoma virus (RSV). We have been investigating this transformation parameter in order to understand at a molecular level some of the events that result in the abnormal properties of neoplastic cells.

Several laboratories have reported a decrease in procollagen synthesis in various transformed cell lines (Green et al., 1966; Hata & Peterkofsky, 1977; Levinson et al., 1975; Kamine & Rubin, 1977; Peterkofsky & Prather, 1974; Schwartz et al.,

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[1] Abbreviations used: RNA, ribonucleic acid; mRNA, messenger RNA; RSV, Rous sarcoma virus; CEF, chick embryo fibroblasts; RSV–CEF, RSV-transformed CEF; ts, temperature sensitive; SR, Schmidt-Ruppin strain of RSV; BH, Bryan high-titer strain of RSV; DBM, diaminobenzyloxymethyl; SSC, 0.15 M sodium chloride and 0.015 M sodium citrate; $NaDodSO_4$, sodium dodecyl sulfate; cDNA, complementary deoxyribonucleic acid; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; poly(A), poly(adenylic acid).

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