Fate of Nucleotides Bound to Reconstituted F_o–F_1 during Adenosine 5′-Triphosphate Synthesis Activation or Hydrolysis: Role of Protein Inhibitor and Hysteretic Inhibition†

François Penin,* Attilio Di Pietro, Catherine Godinot, and Danièle C. Gautheron

Laboratoire de Biologie et Technologie des Membranes du CNRS, Université Claude Bernard de Lyon, 43 Boulevard du 11 Novembre 1918, F-69622 Villeurbanne, France

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ABSTRACT: The protein ATPase inhibitor entraps about five nucleotides in pig heart mitochondrial F_1, one at least being a triphosphate [Di Pietro, A., Penin, F., Julliard, J. H., Godinot, C., & Gautheron, D. C. (1988) *Biochem. Biophys. Res. Commun. 152*, 1319–1325]. The fate of these nucleotides was studied during ATP synthesis driven by NADH oxidation in reconstituted inverted submitochondrial particles. Iodinated F_1, containing 0.7 mol of endogenous nucleotides/mol, was first loaded with tritiated adenine nucleotides in the presence or absence of the protein inhibitor and then reassociated with F_1-depleted submitochondrial particles (ASU particles) to reconstitute an efficient NADH-driven ATP synthesis. In the absence of the protein inhibitor, 1.7 mol of labeled nucleotides remained bound per mole of reassociated F_1, 0.8–0.9 mol being rapidly exchangeable against medium ADP or ATP, as measured after rapid filtration through nitrocellulose filters. In the presence of the protein inhibitor, as many as 3.25 mol of labeled nucleotides remained bound per mole of reassociated F_1. Under hydrolysis conditions where ATPase activity was highly inhibited, no release of tritiated nucleotide occurred. In contrast, under ATP synthesis conditions where the protonmotive force was generated by NADH oxidation, the progressive reversal of inhibition by the protein inhibitor was correlated to a concomitant release of tritiated nucleotide. When ATP synthesis became fully active, about one nucleotide was completely exchanged whereas more than three nucleotides remained tightly bound and did not appear to be directly involved in ATP synthesis. In addition, this paper also demonstrates that the hysteretic inhibition described for isolated F_1 ATPase activity and due to ADP binding to a regulatory site [Di Pietro, A., Penin, F., Godinot, C., & Gautheron, D. C. (1980) *Biochemistry 19*, 5671–5678] is maintained after reassociation of F_1 with ASU particles.

The natural protein inhibitor discovered by Pullman and Monroy (1963) inhibits the mitochondrial ATPase[1] activity of isolated or membrane-bound F_1 [for a recent review, see Schwerzmann and Pedersen (1986)]. The protein inhibitor decreases the exchange of free nucleotides against nucleotides bound to either submitochondrial particles (Harris et al., 1977) or isolated F_1 (Klein et al., 1981). Recent data from our laboratory have shown that when the protein inhibitor inhibits ATPase activity of isolated F_1, up to five nucleotides are firmly bound to the enzyme, one at least of these nucleotides being a triphosphate (Di Pietro et al., 1988). In the present work, the question we ask is: what happens to these nucleotides during ATP hydrolysis or ATP synthesis in reconstituted mitochondrial membranes?

The protein inhibitor does not inhibit the steady-state rate of ATP synthesis in submitochondrial particles, but a lag phase is often observed in the onset of ATP synthesis initiated with a respiratory substrate (Gomez-Puyou et al., 1979; Harris et al., 1979; Schwerzmann & Pedersen, 1981). Controversial interpretations have been proposed to explain the reversal of inhibition in terms of release or no release of the protein inhibitor [Schwerzmann and Pedersen (1981) and for a review Schwerzmann and Pedersen (1986) and Lippe et al. (1988a,b)]. Our purpose was to study whether the lag phase observed in ATP synthesis in the presence of the protein inhibitor was correlated to the specific release of adenine nucleotides in the reconstituted system.

For precise determination of the fate of nucleotides, F_1 purified from pig heart mitochondria was loaded with labeled nucleotides in the presence or absence of the protein inhibitor. Then F_1 was reassociated with F_1-depleted membranes (ASU particles) to reconstitute efficient ATP synthesis driven by NADH oxidation (Penin et al., 1986). A technique of rapid filtration through nitrocellulose filters was developed to titrate the labeled nucleotides remaining bound to membrane-reassociated F_1 during the course of ATP hydrolysis or net ATP synthesis.

One difficulty in evaluating inhibitions due to the protein inhibitor comes from the hysteretic inhibition of F_1 ATPase activity due to ADP binding (Di Pietro et al., 1980) to a regulatory site located on one β subunit (Fellous et al., 1984). Both hysteretic and protein inhibitor inhibitions are additive (Di Pietro et al., 1986, 1988). For reassociation of F_1 to depleted submitochondrial particles, F_1 is first preincubated with 0.5 mM Mg-ATP during 30 min. This preincubation period leads to ATP hydrolysis and thus to the binding of ADP to the regulatory site, leading to hysteretic inhibition of ATPase activity. The present paper indicates that this inhibition is maintained after reconstitution of F_1 to the membranes.

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[1] Abbreviations: ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3); F_1, soluble mitochondrial ATPase; F_o–F_1, mitochondrial ATPase–ATP synthase complex; ASU particles, inverted submitochondrial particles treated with ammonia, Sephadex, and urea to remove F_1; OSCP, oligomycin sensitivity conferring protein; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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Other main results concern the release of about one adenine nucleotide when full activation of ATP synthesis is obtained with NADH oxidation in the presence of the protein inhibitor. However, more than three nucleotides remain bound to reassociated F_1. Under ATP hydrolysis conditions, nucleotides are not released while the inhibition by the protein inhibitor is not reversed; therefore, more than four nucleotides remain bound. In the absence of the protein inhibitor, most nucleotides are immediately exchanged against medium nucleotides whether the enzyme works or not.

Experimental Procedures

Materials. NADH, ADP, ATP, and oligomycin were from Boehringer, and hexokinase (F 300) was from Sigma Chemical Co. Carrier-free [^{32}P]P_i (10 mCi/mL) and Na^{125}I (200 mCi/mL) were from the Commissariat à l'Energie Atomique, France. [2,8-^{3}H]ATP (44 Ci/mmol) was from Amersham. Soluene-350 and Insta-gel were from Packard. Millititer-HA 96-well filtration plates equipped with HA-nitrocellulose filters (0.45-μm pore size) were from Millipore.

Biological Preparations. Previously described procedures were used to prepare pig heart mitochondria (Gautheron et al., 1964; Godinot et al., 1969), ASU particles (Penin et al., 1986), purified F_1 (nucleotide depleted; Penin et al., 1979), purified OSCP (Archinard et al., 1986), and purified protein inhibitor (Di Pietro et al., 1988).

Iodinated F_1 (645 cpm/μg of F_1) was prepared as previously (Penin et al., 1986): an average value of 0.5 mol of ^{125}I was covalently linked per mole of F_1. It has been checked that iodinated F_1 behaved exactly as unlabeled F_1: nucleotide binding and release, ATPase activity, reconstitution, and ATP synthesis were not modified. Iodinated F_1 at 5.2 mg of protein/mL of 50% glycerol, 5 mM EDTA, and 100 mM Tris (sulfate), pH 8.0, was kept frozen in liquid nitrogen. It contained 0.7 mol of endogenous adenine nucleotides (half ATP, half ADP) per mole, as determined by HPLC on Partisil PXS (10-25) SAX (Whatman) after thermal denaturation of the enzyme (Di Pietro et al., 1988).

Loading of F_1 with Tritiated Adenine Nucleotides. Just before use, samples of iodinated F_1 (pH 8.0) were thawed and equilibrated at pH 6.7 by filtration–centrifugation through Sephadex G-50 (fine) columns (Penefsky, 1977) in 25 mM Mops (Tris), 0.25 M sucrose, 10% glycerol, and 1.5 mM $MgSO_4$. The enzyme (1.4 mg of protein/mL) was incubated in the presence or absence of the protein inhibitor (10 mol/mol of iodinated F_1) for 30 min at 30 °C after addition of 0.5 mM [^{3}H]ATP. Free nucleotides were eliminated by filtration–centrifugation through a Sephadex G-50 (fine) column equilibrated in 50 mM Tris (acetate), 0.25 M sucrose, and 1.5 mM $MgSO_4$, pH 7.5.

Reconstitution Procedure. The reconstitution conditions previously described (Penin et al., 1986) were adapted as follows: 530 μg of iodinated F_1 preloaded with tritiated adenine nucleotides in the presence or absence of the protein inhibitor was mixed with pure OSCP (30 μg) and incubated for 30 min at 30 °C with ASU particles (1.6 mg) in 1.1 mL of 50 mM Tris (acetate), pH 7.5, 0.25 M sucrose, 5 mM DTT, and 2.5 mM $MgSO_4$ (final concentrations). The mixture was then centrifuged as 175-μL fractions in a Beckman Airfuge at 25 psi (140000g) for 5 min. The supernatant fluids were removed, and each pellet was rinsed twice with 0.25 M sucrose, 1.5 mM $MgSO_4$, and 50 mM Tris (acetate), pH 7.5, and kept at 30 °C. Each pellet was homogenized at 1 mg of protein/mL of the above buffer just before dilution in the assay media.

Titration of Labeled Nucleotides Remaining Bound to Reconstituted Membranes during the Course of ATP Hydrolysis or ATP Synthesis Driven by NADH Oxidation. Reconstituted membranes were 10-fold diluted and incubated either in the ATP hydrolysis medium [0.25 M sucrose, 1 mM EDTA, 5 mM $MgSO_4$, 3 mM ATP, 50 mM Tris (acetate), pH 7.5] or in the complete ATP synthesis medium [0.25 M sucrose, 1 mM EDTA, 5 mM $MgSO_4$, 1.5 mM NADH, 1 mM ADP, 20 mM potassium phosphate, 50 mM glucose, 40 units of hexokinase/mL, 50 mM Tris (acetate), pH 7.5]. Controls were performed either in the ATP synthesis medium lacking NADH or in a medium without any nucleotide [0.25 M sucrose, 1 mM EDTA, 5 mM $MgSO_4$, 50 mM Tris (acetate), pH 7.5]. At intervals, 225-μL samples were submitted to rapid filtration under vacuum through nitrocellulose filters on a Millititer-HA 96-well filtration plate; the vacuum was regulated to obtain complete filtration in less than 6 s. The filter of each well was cut off and counted for its content in iodinated F_1 in a γ counter (Packard). It was then dissolved overnight in 250 μL of Soluene-350; 4 mL of Insta-gel scintillation medium (or alternatively Ready-solv EP, Beckman) was then added and mixed with 30 μL of 6 N HCl. Under these conditions, counting of ^{3}H-labeled nucleotides could be performed 6 h later when all the chemiluminescence had disappeared. The presence of iodine-125 in the tritium channel was estimated in parallel experiments by scintillation counting of nitrocellulose filters containing known amounts of iodinated F_1 in the absence of tritium label; each value of tritium counting was corrected accordingly. Routinely, the specific radioactivities of iodinated F_1 and of ^{3}H-labeled nucleotides were chosen in such a way that the number of iodine-125 cpm did not exceed 10% of the total cpm in the tritium channel. The HA-nitrocellulose filters used were highly efficient in adsorbing isolated F_1 (97% retention) as well as F_1 reassociated to ASU particles (99.7% retention); less than 2% free labeled nucleotides were unspecifically adsorbed to the filters.

Assay Procedures. ATPase activity was measured spectrophotometrically in the presence of an ATP regenerating system coupled to NADH oxidation (Pullman et al., 1960) in 50 mM Tris (maleate) buffer, pH 8.0, as detailed previously (Godinot et al., 1979) in the presence of 1.5 mM KCN and with or without 1 μg of oligomycin per assay. Net ATP synthesis was measured at 30 °C (Penin et al., 1986) in the complete ATP synthesis medium described above, in the presence of 2×10^6 cpm of [^{32}P]P_i/μmol of P_i. [^{32}P]P_i–ATP exchange activity was measured as previously (Gautheron et al., 1986). Protein contents were estimated by the method of Lowry et al. (1951) as modified by Bensadoun and Weinstein (1976). The molecular masses of F_1 and of the protein inhibitor were taken as 380000 Da (Di Pietro et al., 1975) and 9600 Da (Frangione et al., 1981), respectively.

Results

Effects of the Protein Inhibitor on the Retention of Nucleotides to F_1 after Reconstitution of a Functional F_o–F_1 Complex. Figure 1 shows that iodinated F_1 containing 0.7 mol of endogenous nucleotides bound 3.5 or 4.6 mol of nucleotides when incubated with Mg-[^{3}H]ATP for 30 min in the absence or presence of the protein inhibitor, respectively (step I). Parallel experiments performed with [α-^{32}P]ATP instead of [^{3}H]ATP have shown that, in the absence of the protein inhibitor, all labeled nucleotides bound to F_1 are ADP whereas among the 4.6 labeled nucleotides bound to F_1 in the presence of the protein inhibitor 1.3 are ATP and 3.3 are ADP. In both cases, 0.7 endogenous nucleotide (half ADP, half ATP) remained bound to F_1 (Di Pietro et al., 1988).

After the reconstitution step (step II) (which required 30 min to be efficiently achieved) the same amount of labeled

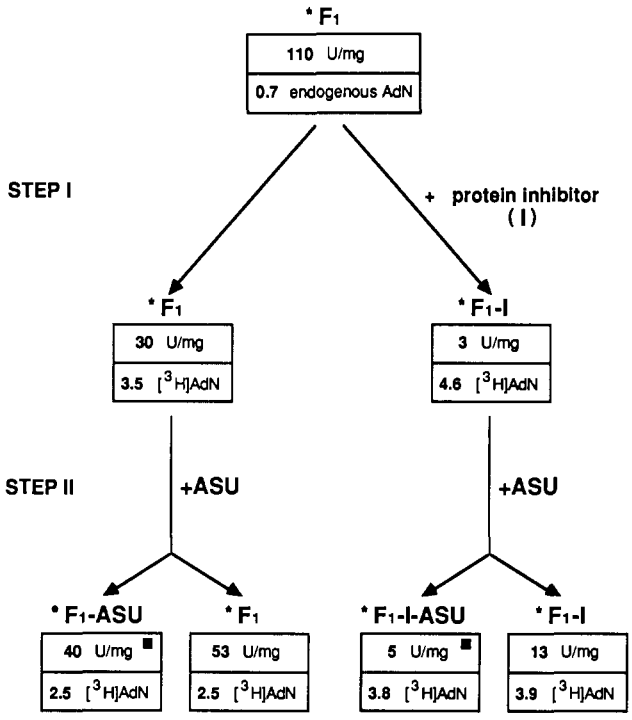

FIGURE 1: ATPase activity and adenine nucleotides bound to iodinated F_1 before and after reconstitution with ASU particles. Effects of the protein inhibitor. $*F_1$, iodinated F_1; I, protein inhibitor; AdN, adenine nucleotides. (Step I) Loading with labeled nucleotides. $*F_1$ (245 cpm/pmol of F_1) was loaded with tritiated nucleotides by incubation with 0.5 mM [^{3}H]ATP (940 dpm/pmol) at pH 6.7 for 30 min in the presence or absence of the protein inhibitor as described under Experimental Procedures. After filtration–centrifugation at pH 7.5, one aliquot of the first eluate was submitted to a second filtration–centrifugation for complete removal of free nucleotides; ATPase activity and bound ^{3}H-labeled nucleotides on $*F_1$ in the presence of the protein inhibitor ($*F_1$–I) or in its absence ($*F_1$) were estimated on the second eluate. (Step II) Reconstitution. The remaining eluate from the first centrifugation–elution was incubated for 30 min with ASU particles (as described under Experimental Procedures) to reconstitute the F_o–F_1 complex containing the protein inhibitor ($*F_1$–I–ASU) or not ($*F_1$–ASU). Free $*F_1$ or $*F_1$–I (140000g supernatant) was submitted to filtration–centrifugation (pH 7.5) as above; ATPase activity and the remaining bound ^{3}H-labeled nucleotides were measured in the eluate. The reconstituted particles (140000g pellet) were homogenized in 0.25 M sucrose, 1.5 mM $MgSO_4$, and 50 mM Tris (acetate), pH 7.5, and counted to estimate reassociated $*F_1$ and ^{3}H-labeled nucleotides amounts. ATPase activity is expressed as units per milligram of protein; 1 unit corresponds to 1 μmol of ATP hydrolyzed/min. Bound nucleotides are expressed as moles per mole of $*F_1$. The experimental precision was in the range of 3–5%. The symbol (■) indicates that ATPase activity was sensitive to oligomycin (at least 92%).

nucleotides remained bound to F_1 whether F_1 (or F_1–I in Figure 1) was reassociated with ASU particles or was in solution. In the presence of the protein inhibitor, 3.8 mol of labeled nucleotides/mol of F_1 remained bound to the reconstituted F_o–F_1 complex (F_1–I–ASU in Figure 1) while 2.5 mol remained bound in the absence of the protein inhibitor (F_1–ASU in Figure 1). In both cases, about 1 mol of labeled nucleotides was released during this step.

Evolution of ATPase Activity during the Reconstitution Procedure. The iodination of F_1 did not significantly modify its specific ATPase activity (110 units/mg of protein). Figure 1 shows that during the loading of F_1 with labeled nucleotides (step I, 30-min incubation with Mg-[^{3}H]ATP) the ATPase activity of F_1 decreased from 110 to 30 units/mg. This inhibition was due to the binding to the regulatory site of ADP produced by ATP hydrolysis and was of the hysteretic type, as previously described (Di Pietro et al., 1980). This hysteretic inhibition was barely reversed during the reconstitution step with ASU particles (step II). Indeed, the ATPase activity of soluble F_1 reached 53 units/mg of F_1 while that of reassociated F_1–ASU was only 40 units/mg of F_1. Therefore, the ADP-induced inhibition was maintained during the reassociation of F_1 with ASU particles.

The ATPase activity of F_1 was further decreased up to 3 units/mg when the protein inhibitor was present during step I. This inhibition was essentially maintained during the reconstitution of the F_1–inhibitor complex with ASU (step II) since the ATPase activity of soluble F_1–inhibitor or of reassociated F_1–inhibitor–ASU (respectively F_1–I and F_1–I–ASU in Figure 1) was of 13 or 5 units/mg, respectively.

Effects of the Protein Inhibitor on the Release of Labeled Nucleotides Bound to Reassociated F_1 during ATP Hydrolysis or ATP Synthesis Driven by NADH Oxidation in Reconstituted Membranes. When reconstituted F_1–ASU particles containing 2.45 mol of ^{3}H-labeled nucleotides/mol of F_1 (as shown in Figure 1) were 10-fold diluted in a medium devoid of nucleotides in view of measuring activities, only 1.7 mol of labeled nucleotides remained bound (Figure 2A, empty squares). This residual amount was stable during several minutes of incubation. On the contrary, an additional amount of 0.8–0.9 mol of ^{3}H-labeled nucleotides/mol of F_1 was exchanged in less than 15 s (the shortest time experimentally tested) in any medium containing nucleotides: ATP hydrolysis medium (closed triangles), ATP synthesis medium lacking NADH (closed squares), or complete ATP synthesis medium (open circles). In the latter case, out of the 0.8 mol of ^{3}H-labeled nucleotides remaining bound, only 0.1 mol was slowly exchanged over 6 min. Figure 2B shows that during these 6 min a linear synthesis of ATP was observed with a constant rate of 2.15 μmol of ATP synthesized min^{-1} (mg of reassociated F_1)$^{-1}$.

When F_1–inhibitor–ASU particles containing 3.8 mol of ^{3}H-labeled nucleotides per mole of F_1 (star, Figure 3A) were 10-fold diluted, as much as 3.25 mol remained bound to F_1 (Figure 3A). In contrast to the preceding results in the absence of the protein inhibitor, no rapid exchange was observed here when the 6-min incubation was performed in the ATP hydrolysis medium (closed triangles) or in the ATP synthesis medium lacking NADH (closed squares). It should be mentioned that ATP hydrolysis remained inhibited (more than 90%) under these conditions (data not shown). Other experiments not reported here have shown that under [^{32}P]P_i–ATP exchange conditions neither ATP synthesis nor release of labeled nucleotides was significantly detected.

A significant exchange of ^{3}H-labeled nucleotides was only observed under ATP synthesis conditions (open circles, Figure 3A). This exchange was slow and reached a maximal extent of 0.6 mol of ^{3}H-labeled nucleotides/mol of reassociated F_1 over 5 min of ATP synthesis. After this time, as many as 2.65 mol of ^{3}H-labeled nucleotides remained bound to reassociated F_1. Figure 3B shows that the low initial rate of ATP synthesis was progressively activated as a function of incubation time. A constant rate of about 1.85 μmol of ATP synthesized min^{-1} (mg of reassociated F_1)$^{-1}$ was reached after 5 min. This rate of ATP synthesis was close to that observed in the absence of the protein inhibitor (Figure 2B).

The activation of ATP synthesis rate (Figure 3B) and the release of bound ^{3}H-labeled nucleotides during ATP synthesis (open circles, Figure 3A) followed the same first-order kinetics (not shown) with a rate constant of 8.5×10^{-3} s^{-1}, giving a half-time of 1.9 min.

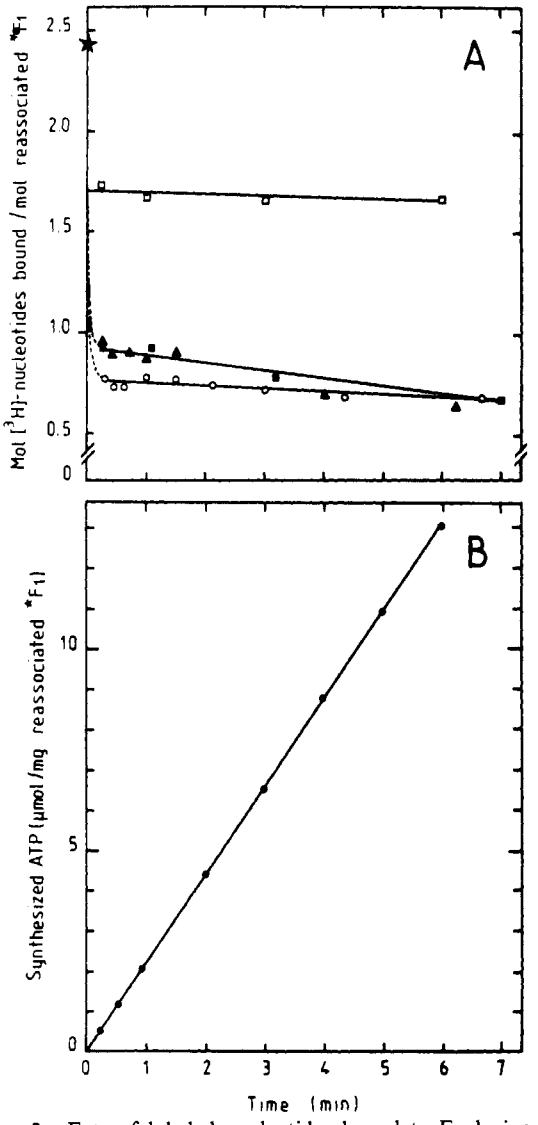

FIGURE 2: Fate of labeled nucleotides bound to F_1 during ATP hydrolysis or ATP synthesis driven by NADH oxidation in reconstituted particles. Iodinated F_1 ($*F_1$) was loaded with tritiated nucleotides and reassociated with ASU particles as described in Figure 1. Reconstituted particles were homogenized at 1 mg of protein/mL and 10-fold diluted at $t = 0$ into various assay media (see below). (A) Kinetics of release of tritiated nucleotides: The star (★) in the ordinate axis was the molar ratio of ^3H-labeled nucleotides bound to reassociated $*F_1$ before 10-fold dilution into assay media. The nucleotides remaining bound to $*F_1$ were titrated at the indicated times after rapid filtration of samples through nitrocellulose filters on Millititer-HA plate. The molar ratio of ^3H-labeled nucleotides remaining bound to reassociated $*F_1$ was measured on filters as described under Experimental Procedures. Assay media: complete ATP synthesis medium (○) and control medium without NADH (■); ATP hydrolysis medium (▲); control medium without any nucleotide (□). The composition of these media is described under Experimental Procedures. (B) Kinetics of ATP synthesis driven by NADH oxidation: Reconstituted particles were 10-fold diluted at $t = 0$ into the same complete ATP synthesis medium as in (A), except that $[^{32}P]P_i$ was present (1685 cpm/nmol). Aliquot samples were withdrawn at the indicated times to measure the amount of synthesized $[\gamma^{32}P]ATP$ (see Experimental Procedures).

Correlation between the Activation of ATP Synthesis Driven by NADH Oxidation and the Release of F_1-Bound Nucleotides in Reconstituted Particles in the Presence of the Protein Inhibitor. Figure 4 shows that, all along the phase of ATP synthesis activation in the presence of the protein inhibitor, the rate of ATP synthesis was linearly correlated to the release of bound ^3H-labeled nucleotides. Extrapolation to the abscissa axis indicates that the initial ATP synthesis rate would be completely inhibited when about 0.85 mol of nucleotides was bound per mole of reassociated F_1 (0.55 measured + 0.3 ex-

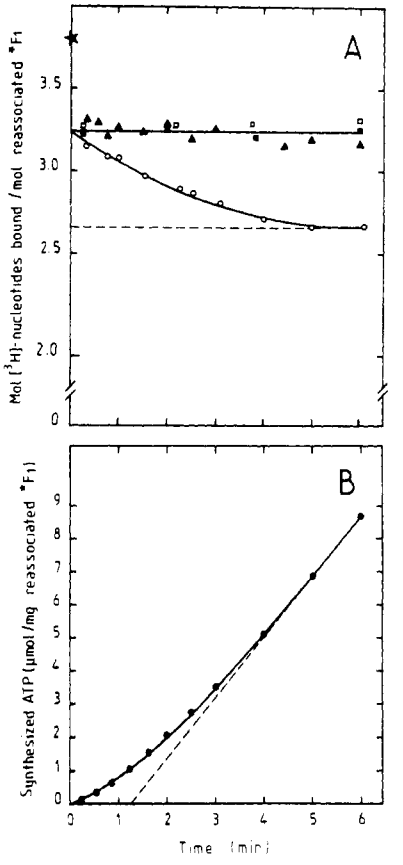

FIGURE 3: Fate of nucleotides bound to F_1 in the presence of the protein inhibitor during ATP hydrolysis or ATP synthesis driven by NADH oxidation in reconstituted particles. Iodinated F_1 ($*F_1$) was incubated with the protein inhibitor, loaded with tritiated nucleotides, and reassociated to ASU particles under the conditions described in Figure 1. All other conditions were the same as those detailed in the legend of Figure 2. The dotted lines represent the final level of ^3H-labeled nucleotides remaining bound under ATP synthesis conditions (A) or steady-state synthesis of ATP (B).

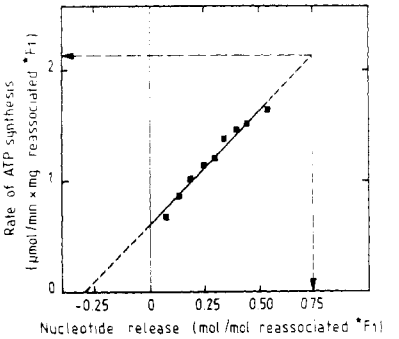

FIGURE 4: Correlation between ^3H-labeled nucleotide release and the rate of ATP synthesis during the activation phase of ATP synthesis in the presence of the protein inhibitor. From data of Figure 3B, the rate of ATP synthesis was graphically estimated by the slope of the curve at each indicated time, up to 5 min. The values obtained were plotted against the corresponding values of nucleotide release. The latter was calculated from the data of Figure 3A (empty circles) by the difference between ^3H-labeled nucleotides remaining bound at each time and initial ^3H-labeled nucleotide binding. Solid line is calculated by linear regression analysis ($R = 0.97$). Dotted line in the left quadrant extrapolates to a null rate of ATP synthesis. The arrows at the end of the dashed lines in the right quadrant indicate the amount of nucleotide to be released to reach the rate of ATP synthesis obtained with reassociated $*F_1$ in the absence of the protein inhibitor (2.15 μmol of ATP synthesized min^{-1} mg^{-1}; data from Figure 2B).

trapolated). If one extrapolates the amount of nucleotides that would be released to reach the rate of ATP synthesis observed in the absence of the protein inhibitor [2.15 μmol of ATP min^{-1} (mg of reassociated F_1)$^{-1}$, Figure 2B], about 1 mol of nucleotide released per mole of F_1 can be estimated.

Discussion

This paper demonstrates that upon energization of membrane-bound F_o–F_1 by the respiratory chain the reversal of the inhibition of ATP synthesis by the protein inhibitor is kinetically correlated to the release of one initially entrapped adenine nucleotide.

This correlation could be established by combination of several experimental approaches:

(i) Reconstitution of F_o–F_1 complex efficient for catalysis of high ATP synthesis driven by NADH oxidation [2.15 μmol of ATP min^{-1} (mg of reassociated F_1)$^{-1}$] was performed with F_1-depleted submitochondrial particles, OSCP, and F_1 (containing only 0.7 mol of endogenous nucleotides) preloaded with [^{3}H]adenine nucleotides in the presence or absence of the protein inhibitor.

(ii) The use of iodinated F_1 in every experiment allowed us to accurately estimate the amount of reassociated F_1, the molar ratio of ^{3}H-labeled nucleotides bound to reassociated F_1, and the specific ATP synthesis activity of reassociated F_1.

(iii) A technique of rapid filtration through nitrocellulose filters has been developed to measure labeled nucleotides remaining bound to F_1 after reassociation to the membranes. The method presents a number of advantages: complete retention of the membranes on the filters, very efficient elimination of free nucleotides in the filtrates, and complete recovery of tritium radioactivity after solubilization of the nitrocellulose filters and counting. The Millititer apparatus (Millipore) permits separation of the membranes from the assay medium in a few seconds, and the 96-well plates allow us to perform simultaneously many assays for kinetic purposes. Therefore, the fate of [^{3}H]adenine nucleotides preloaded on F_1 could be followed after reconstitution of F_o–F_1 complex and during its turnover for ATP hydrolysis or ATP synthesis.

When isolated F_1 ATPase activity is inhibited by the protein inhibitor, five nucleotides (0.7 endogenous) are entrapped in F_1, one at least being a triphosphate. It is shown here that as many as 3.25 mol of labeled nucleotides (in addition to the 0.7 endogenous) remains bound to F_1 after reassociation with depleted membranes and dilution into various media. These entrapped nucleotides cannot be exchanged against added cold nucleotides or under ATP hydrolysis conditions. On the contrary, in the absence of the protein inhibitor, only 1.7 mol of labeled nucleotides (instead of 3.25) remains bound after reconstitution and dilution; 0.9 mol of these nucleotides is immediately exchanged against any medium containing nucleotides. It can be concluded that the presence of the protein inhibitor during reconstitution experiments is a good tool to retain nucleotides on F_1 and to study their behavior during oxidative phosphorylation.

It is worth mentioning here experiments from Penefsky (1985). He observed the formation of [^{32}P]glucose 6-phosphate in the presence of hexokinase upon energization of beef heart KCl-treated submitochondrial particles preloaded with the limitant [γ-^{32}P]ATP. On this basis, he concluded that "about one third of the bound radioactive ATP appears to dissociate ... from the high affinity catalytic site". However, no direct titration of ATP or other nucleotide was performed either in the presence or in the absence of the protein inhibitor, during ATP synthesis or hydrolysis. Maybe the immediate release of one nucleotide observed in our controls made in the absence of the protein inhibitor (Figure 2A and just above), after addition of ADP or of the full medium to initiate ATP synthesis or hydrolysis, could be related to the dissociation of ATP reported by Penefsky. However, since the nucleotide in our experiments is released under noncatalytic as well as catalytic conditions, we cannot conclude on the involvement of a catalytic site.

Many experiments with submitochondrial particles have shown a latency in the activation of ATP synthesis upon addition of a respiratory substrate. The involvement of the protein inhibitor in this activation is still controversial [for a review, see Schwerzmann and Pedersen (1986)]. Our results clearly show that this activation phase is observed only when the protein inhibitor is present. The activation of ATP synthesis with our reconstituted membranes obeys first-order kinetics, as also observed in submitochondrial particles (Husain et al., 1985). However, the activation process is much longer with our reconstituted membranes in correlation with the slow establishment of the protonmotive force (Penin et al., 1986). The half-time of activation, 1.9 min, is long enough to allow us to easily follow the increase of the rate of ATP synthesis as well as the concomitant release of labeled nucleotides. The present results (Figure 4) clearly demonstrate that a direct correlation can be established between the activation of ATP synthesis and the release of about 1 mol of labeled nucleotide/mol of reassociated F_1. In conclusion, 1 mol of nucleotide entrapped in F_1 in the presence of the protein inhibitor must be released to reach the steady-state rate of ATP synthesis, similar to that immediately obtained in the absence of the protein inhibitor.

The only way to produce the above specific ^{3}H-labeled nucleotide release correlated to the activation of net ATP synthesis is to generate a protonmotive force by respiratory substrate oxidation, here NADH. The presence of 3–10 mM ATP, even if ATPase is not fully inhibited by the protein inhibitor, cannot produce the specific release of nucleotides and the activation of [^{32}P]P_i–ATP exchange. These data are in agreement with Lippe's et al. (1988b) results which show that ATP prevents the reversal of inhibition by the protein inhibitor.

Another main result of this work is that as many as 3.35 mol of nucleotides (2.65 labeled + 0.7 endogenous) per mole of reassociated F_1 in the presence of the protein inhibitor remains bound and not exchangeable after the maximal ATP synthesis turnover is reached. Therefore, these nucleotides behave as tightly bound nucleotides and appear not to be directly involved in ATP synthesis. It should be stressed that the ADP-induced hysteretic inhibition of isolated F_1 ATPase activity (Di Pietro et al., 1980) is maintained during reconstitution experiments, demonstrating that this hysteretic inhibition is also observed with membrane-bound F_1. Therefore, the ADP regulatory site must be occupied even when the full rate of ATP synthesis is reached. Besides, our experiments do not exclude that the regulatory site remains occupied in the absence of the protein inhibitor.

In conclusion, our data indicate that the generation of the protonmotive force by NADH oxidation, which reverses the inhibition due to the protein inhibitor, induces the release of one nucleotide from membrane-bound F_1. One can wonder whether this nucleotide release is the primary event during ATP synthesis or if the release follows events related to the protein inhibitor: conformational changes, displacement, or unbinding from membrane-bound F_1.

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Registry No. ATP, 56-65-5; ADP, 58-64-0; ATPase, 9000-83-3; ATP synthase, 37205-63-3.

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Nuclear Envelope Glycoprotein with Poly(A) Polymerase Activity of Rat Liver: Isolation, Characterization, and Immunohistochemical Localization†

Rabinder N. Kurl, Stephanie C. Holmes, Ethel Verney, and Herschel Sidransky*

Department of Pathology, The George Washington University Medical Center, Washington, D.C. 20037

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ABSTRACT: A protein with poly(A) polymerase activity has been identified and isolated from hepatic nuclear envelopes of rats to near homogeneity. The ability of the enzyme to bind to concanavalin A–agarose and to be eluted from the column with methyl α-D-mannopyranoside (0.2 M) as well as the inhibitory effects of α-mannosidase suggested that it was a glycoprotein. Poly(A) polymerase has an absolute requirement for a divalent cation, ATP, and an oligonucleotide primer. The enzyme activity with Mn^{2+} was about 20-fold higher than that with Mg^{2+}. Several known inhibitors adversely affected poly(A) polymerase activity. The enzyme has a molecular weight of 64 000 when analyzed by polyacrylamide gel electrophoresis under denaturing conditions and has a sedimentation coefficient of 4.5 S. Immunohistochemical studies using polyclonal antibodies raised against the purified enzyme revealed that the antigen was localized in the nuclear membranes.

Polyadenylation is one of the nuclear processes involved in the maturation of hnRNA prior to its transport to the cytoplasm as mRNA. Poly(A) sequences have been implicated in (i) mRNA stability (Nudel et al., 1976), (ii) nucleocytoplasmic transport of mRNA (Muller et al., 1984), and (iii) protein synthesis (Jacobson & Favreau, 1983; Rubin & Halim, 1987). However, most histone mRNAs lack poly(A) sequences (Adesnik & Darnell, 1972; Greenberg & Perry, 1972), and also a population of mRNA isolated from adult rat brains has recently been shown to lack the sequences (Chaudhari & Hahn, 1983).

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