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Proton–Adenosinetriphosphatase Complex of Rat Liver Mitochondria: Effect of Energy State on Its Interaction with the Adenosinetriphosphatase Inhibitory Peptide†

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ABSTRACT: The effect of energy state on the capacity of the H^+–ATPase of inverted inner membrane vesicles of rat liver mitochondria to interact with a homogeneous inhibitor peptide from the same source [Cintron, N. M., & Pedersen, P. L. (1979) *J. Biol. Chem. 254*, 3439–3443] has been examined in some detail. The study has been conducted by using an assay procedure which allows both ATP synthetic and hydrolytic activities of the H^+–ATPase to be monitored in the same assay system. When the purified inhibitor is incubated with inverted inner membrane vesicles in the presence of MgATP and then sedimented to remove excess inhibitor and MgATP, the ATPase activity of the H^+–ATPase is markedly inhibited. When ATP synthesis is induced in the same assay system by initiation of respiration (addition of succinate), the synthetic rate proceeds with a brief lag phase, in the order of seconds, and then assumes a linear steady-state rate. Under these conditions, most of the peptide inhibitor remains associated with the inner membrane vesicles. It is released into the supernatant only when respiration is allowed to proceed several minutes. Inhibitor release is accompanied by a parallel rise in the capacity of the H^+–ATPase to catalyze ATPase activity under nonenergized conditions (succinate absent). These results emphasize that binding of the peptide inhibitor to the H^+–ATPase complex of rat liver and its release therefrom correlate well with the capacity of the enzyme to catalyze ATP hydrolysis rather than ATP synthesis. The lag phase in ATP synthesis when inhibitor is present is very brief and may reflect the time required for a respiration-induced electrochemical gradient to weaken the binding of the inhibitor to the enzyme surface. It would seem that if the peptide inhibitor is a regulatory molecule, one of its major roles in intact rat liver mitochondria may be to preserve newly synthesized ATP following a burst of "phosphorylating" respiration.

ATPase inhibitor peptides have been isolated from mitochondria (Pullman & Monroy, 1963; Horstman & Racker, 1970; Satre et al., 1975; Chan & Barbour, 1976a; Ebner & Maier, 1977; Cintrón & Pedersen, 1979; Yamada et al., 1980), from chloroplasts, (Nelsen et al., 1972; Younis et al., 1980), and from bacteria (Smith & Sternweis, 1977). In all cases, they have been shown to be potent inhibitors of the ATP hydrolytic activity catalyzed by H^+–ATPases (F_0F_1-ATPases) associated with these systems. These inhibitors have been studied in detail with respect to their physicochemical properties. Without exception, they are small molecules ranging from 5000 to 16000 daltons. They constitute either the smallest of the five types of subunits characteristic of the F_1 moiety or a distinct "sixth" subunit. In chloroplasts, there have been reports of two inhibitor proteins, one constituting the ϵ or the smallest subunit (Nelson et al., 1972) and another which is distinct from the F_1 subunits (Younis et al., 1980).

To date, the role of these various ATPase inhibitor proteins is not at all clear. Although it has become common to refer to this class of proteins as regulatory molecules, it remains to be established what their regulatory role is. It has been suggested that such inhibitors may regulate the direction of energy flux through H^+–ATPases, i.e., toward ATP synthesis by blocking only the ATP-dependent or ATP hydrolytic activities (Asami et al., 1970). However, it has also been suggested that, depending on the conditions, these inhibitors may regulate and/or inhibit ATP synthesis (Harris & Crofts, 1978; Gómez-Puyou et al., 1979; Harris et al., 1979), the ADP–ATP transport system (Chan & Barbour, 1976b), and even Ca^{2+} uptake (Gómez-Puyou et al., 1980).

Several workers have indicated that inhibitor binding may be dependent on the energy state of the mitochondrion (Van De Stadt et al., 1973; Gómez-Puyou et al., 1979; Harris et al., 1979). In the most recent set of studies along these lines, it has been assumed that the inhibitor is associated with the H^+–ATPase complex in such a way as to block both ATP synthesis and ATP hydrolysis (Gómez-Puyou et al., 1979; Harris et al., 1979). Initiation of respiration is suggested to dissociate the inhibitor so that the electrochemical gradient of protons can drive ATP synthesis (Harris & Crofts, 1978; Gómez-Puyou et al., 1979; Harris et al., 1979). Although these studies certainly provide a novel view of the regulatory nature of peptide inhibitors, they provide no direct evidence (i.e., appearance of the released inhibitor in the supernatant) to support the suggested hypothesis. Rather, increase in ATPase activity is used as an indirect index of inhibitor release.

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Complications in interpretations are further compounded by the fact that ATPase and ATP synthetic activities are monitored in separate assay systems.

In this study, we have focused on one aspect of the peptide inhibitor problem, namely, that dealing with its suggested role as a regulator of the H^+–ATPase. Specifically, we have addressed the question of whether the energy state of inner membrane vesicles influences the inhibitor interaction with the H^+–ATPase and whether inhibitor binding and release from the enzyme correlate with changes in ATPase activity, ATP synthetic activity, or both activities. In order to avoid complications in interpretation hampering previous studies, we have used a system which allows both ATP synthetic and hydrolytic activities to be monitored in the same assay. We have also monitored the release of inhibitory activity into the supernatant rather than assuming that an increase in ATP synthetic or ATPase activity can be equated with inhibitor release.

Experimental Procedures

Materials

Adult male CD albino rats were obtained from the Charles River Breeding Laboratories, Wilmington, MA, and were fed ad libitum a Rockland rat diet purchased from Tekland, Winfield, OH. Sucrose, D(+)-mannitol, sodium succinate, and dinitrophenol (DNP)[1] were from Baker Chemical Co. Oligomycin, Tris, bovine albumin, pyruvate kinase, synthetic luciferin, firefly luciferase (type IV), and ADP (grade III) were from Sigma Chemical Co. Hepes was from Calbiochem, digitonin from Grand Island Biological Co., lactic dehydrogenase from Boehringer Mannheim, and Sephadex G-75 from Pharmacia. ATP and NADH were purchased from P-L Biochemicals. Washed and ignited seasand was from VWR Scientific, Inc. All other reagents were of reagent grade purity.

Methods

Preparation of Mitochondria and Inverted Inner Membrane Vesicles. The mitochondria were isolated from rat liver according to the high-yield procedure of Bustamante et al. (1977) in H medium containing 220 mM D(+)-mannitol, 70 mM sucrose, 5 mM Hepes–KOH, and 0.5 mg of defatted BSA/mL (pH 7.4). Inverted inner membrane vesicles were obtained from mitoplasts by water lysis and subsequent sonication exactly as described by Pedersen et al. (1978). The vesicles were suspended in the mitochondrial isolation medium at 25–40 mg of protein/mL and stored in liquid nitrogen.

Purification of the ATPase Inhibitor. The peptide inhibitor from rat liver mitochondria was purified according to the procedure of Cintrôn & Pedersen (1979) with only minor modifications. DTT was omitted from the phosphate–EGTA buffer used in the heat and chromatographic steps. The chromatography of the heat-treated material was done in a column (480 × 9 mm) containing at the bottom a 5–10-cm layer of seasand. It was found that the inhibitor peptide at this stage adsorbs strongly to the sand and can be eluted in practically pure form with a potassium phosphate buffer (500 mM, pH 5.7) and 1 mM EGTA. The purified protein showed a single band in $NaDodSO_4$–polyacrylamide gel electrophoresis, exactly as described by Cintrôn & Pedersen (1979). The specific activity of the inhibitor peptide ranged from 3000 to 5000 units. One unit is defined as the amount of inhibitor peptide required to inhibit 0.2 unit of ATPase activity.

Preparation of Peptide Inhibitor Supplemented Inner Membrane Vesicles. Inverted inner membrane vesicles (1–2 mg of protein/mL) were incubated with an excess of purified inhibitor (20–40 units/mg of inner membrane vesicles) in a medium containing 250 mM sucrose, 0.5 mM MgATP, and 5 mM imidazole-HCl at pH 6.7. After 15 min at room temperature, the suspension was centrifuged for 30 min at 100000*g*. The sediment was suspended in mitochondrial isolation medium. This treatment yielded vesicles which have a very low ATPase activity (0.20–0.35 μmol of ATP hydrolyzed min^{-1} mg^{-1}) relative to the original ATPase activity (2.5–3.5 μmol of ATP hydrolyzed min^{-1} mg^{-1}). Vesicles subject to the same treatment but in the absence of inhibitor peptide are called control vesicles. Their ATPase activity is unaltered relative to that of the starting material.

Spectrophotometric Assay of ATPase Activity. ATPase activity was measured at 340 nm by coupling the production of ADP to the oxidation of NADH via the pyruvate kinase and lactic dehydrogenase reactions exactly as described previously (Cintrôn & Pedersen, 1979).

Luminescent Assay of ATPase and ATP Synthetic Activities. The synthesis of ATP was measured with the luciferin–luciferase assay in a manner similar to that described by Lemasters & Hackenbrock (1976). The light emission of the reaction was detected with the LKB-Wallac Model 1250 luminometer (Turku, Finland) and recorded on a recorder 2210 from the same manufacturer. Rapid mixing during measurements was achieved by positioning a magnetic stirrer close to the measuring head of the luminometer which activated the magnetic stirring bar in the reaction cuvette. Additions were made with the LKB injection device or with a Hamilton-type syringe equipped with a long needle (SGE, Austin, TX). This assay system allowed continuous measurement and offered a resolution time of less than 1 s. It also permitted the hydrolysis of ATP to be followed as a decrease in light emission as described under Results.

The standard reaction medium contained 0.8 mL of 20 mM Hepes, 10 mM potassium phosphate, 5 mM $MgCl_2$, and 1 mM EGTA, pH 7.5, and 0.1 mL each of luciferin (0.15 mM) and luciferase (0.02 mg/mL) in 5 mM sodium glycylglycine (pH 7.6). The final concentration of ADP was between 1 and 20 μM, depending upon the experiment. The amount of inner membrane vesicles used in this assay ranged from 50 to 200 μg of protein. The synthesis of ATP was usually started by injecting 10 μL of a respiratory substrate (100 mM).

Protein Determination. Protein was determined either by the biuret method (Jacobs et al., 1956) for mitochondria and submitochondrial fractions or by the Lowry et al. (1951) method in the case of soluble proteins.

Results

Characterization of Inner Membrane Vesicles (IMV). In order to study the influence of exogenously added ATPase inhibitor peptide on the ATP hydrolytic and synthetic activities of submitochondrial particles, it is important to know the endogenous inhibitor content. Both indirect and direct measurements indicate that the ATPase peptide inhibitor content of the IMV used in this study is very low. As shown in Table I, incubation of the particles with MgATP does not alter the control rate of ATP hydrolysis. Since MgATP is known to

[1] Abbreviations used: BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone; DNP, dinitrophenol; Cl_3CCOOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

Table I: Characterization of Purified Inner Membrane Vesicles (IMV) of Rat Liver[a]

	ATPase activity (μmol of ATP hydrolyzed min^{-1} mg^{-1})
control	2.54
+MgATP (0.5 mM)	2.41
+NADH (2.5 mM)	2.67

endogenous inhibitor content: 0.3 unit/mg of IMV protein
exogenous inhibitor required for 90% inhibition of ATPase activity: 18 units/mg of IMV

RCI (with FCCP): 2.2

[a] IMV free of outer compartment and matrix components were prepared exactly as described by Pedersen et al. (1978). ATPase activity was measured with the coupled enzyme assay as described under Methods. Prior to assay, IMV were incubated for 10 min at 0.2 mg of protein/mL in 250 mM sucrose and 5 mM imidazole-HCl. The pH was 6.7 when MgATP was present and 7.4 when NADH was present. Endogenous inhibitor content was determined by extracting 50 mg of IMV with alkali exactly as described by Cintrón & Pedersen (1979) for mitochondria and then subjecting the extract to the same heat treatment. The amount of inhibitor activity in the final supernatant was assayed as described under Methods. The respiratory control index (RCI) was determined polarographically in H medium (see Methods) with NADH (1 mM) as substrate and 1 μM FCCP as the uncoupler.

be required for inhibition of ATPase activity in IMV by the peptide inhibitor (Cintrón & Pedersen, 1979), this experiment tends to rule out the possibility that the particles contain "silent" endogenous inhibitor. Energization of the particles by inducing respiration with NADH also has no effect on ATPase activity. Of more significance is the finding that only 0.3 unit of inhibitor activity can be extracted from 1 mg of particles which normally require 18 units of inhibitor activity to suppress the ATP hydrolytic activity more than 90% (see also Figure 2B). The particles used in this study therefore have very little residual inhibitor content and, as also indicated in Table I, exhibit respiratory control.

Assay of ATP Hydrolysis and ATP Synthesis in the Same Assay System. Since the major purpose of this work was to assess the effect of energy state on the interaction of the ATPase peptide inhibitor of rat liver mitochondria with the H$^+$-ATPase from the same source, it seemed essential to be able to assay both the synthesis and hydrolysis of ATP under conditions as identical as possible. As illustrated in Figure 1, this was accomplished by using the firefly assay and automatically monitoring light emission with a luminometer connected to a strip chart recorder (see Methods). Figure 1A shows that upon addition of ATP to the system containing the luciferin–luciferase pair the typical light emission response pattern is observed immediately. In fact, the light emitted was found to be proportional to the amount of ATP present over a wide concentration range (10^{-6}–10^{-10} M). If purified F_1-ATPase is added, a decrease in light emission occurs as ATP is hydrolyzed. The initial rate of ATP hydrolysis is directly proportional to the amount of F_1-ATPase added. (The slow back-decay of the light signal in the absence of added F_1-ATPase is probably due to a contaminating ATPase activity in the luciferase preparation. Different lots of luciferase obtained from the same supplier were found to exhibit different back-decay rates).

Results presented in Figure 1B show that addition of the respiratory substrate succinate to inner membrane vesicles results in a rapid burst of ATP synthesis which can be reversed by adding DNP, which induces ATP hydrolysis. (At the concentration used, DNP induces ATPase activity by acting as an uncoupler, not by chemically modifying the F_1–ATPase complex.) A second ATP synthetic cycle cannot be induced by succinate because DNP has uncoupled respiration from phosphorylation. Addition of ATP provides substrate for another round of ATP hydrolysis which can be blocked by oligomycin, an inhibitor of the H$^+$-ATPase. (The inhibitors at the concentrations used were without effect on the ATP-induced light signal. Light emission was quenched less than 5% by DNP and not at all by oligomycin).

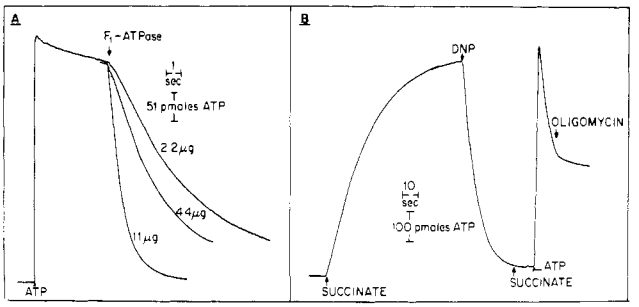

FIGURE 1: Measurement of ATP synthesis and ATP hydrolysis in the luciferin–luciferase system. (A) The reaction medium was as described under Methods. Where indicated, 4 μL of 0.1 mM ATP or indicated amounts of purified F_1-ATPase (Catterall & Pedersen, 1971) was added. (B) The assay system was exactly as described in (A) with 75 μg of inner membrane vesicles. Where indicated, 10 μL of 100 mM succinate, 5 μL of 10 mM DNP, 10 μL of 0.1 mM ATP, or 1 μg of oligomycin was added.

Taken together, these experiments demonstrate clearly that the synthesis and hydrolysis of ATP can be monitored rapidly and very sensitively in the luminescent firefly assay system. Significantly, energized conditions (ADP, P_i, and respiratory substrate present) induce ATP synthesis, whereas nonenergized conditions (uncoupler or respiratory inhibitors present) induce ATP hydrolysis.

Capacity of H$^+$-ATPase To Catalyze ATP Hydrolysis in the Presence and Absence of the ATPase Peptide Inhibitor: Effect of Energy State. Results presented in Figure 2 show that prior incubation of inner membrane vesicles of rat liver mitochondria with the purified ATPase inhibitor peptide from the same source (see Methods) results in a marked inhibition of the rate of hydrolysis of either added ATP (compare the light decay responses in Figure 2A,B) or newly synthesized ATP (compare the light decay response after addition of DNP in Figure 2C,D). As previously reported with less sensitive assay procedures (Pullmann & Monroy, 1963; Horstman & Racker, 1970), the steady-state rate of oxidative phosphorylation is seen to be unaffected by the inhibitor peptide (compare the light emission response in Figure 2C,D after addition of succinate).

Results presented in Figure 3A,B summarize the effect of energy state on the capacity of the H$^+$-ATPase to catalyze ATP hydrolysis in untreated vesicles and in vesicles treated with purified ATPase peptide inhibitor. Vesicles, either without inhibitor or supplemented with exogenously added inhibitor, were allowed to carry out oxidative phosphorylation for different intervals of time (i.e., they were subjected to "energized conditions" for different time intervals). ATP hydrolysis was then induced with DNP which transforms the energy state immediately from "energized" to "nonenergized". As can be seen in Figure 3A, vesicles which have not been pretreated with inhibitor have the same rate of ATP hydrolysis regardless of the amount of time to which they have been subjected to energized conditions. In contrast, vesicles which have been pretreated with inhibitor require almost 12-min incubation under energized conditions to attain the same capacity for ATP hydrolysis (under nonenergized conditions) as untreated vesicles.

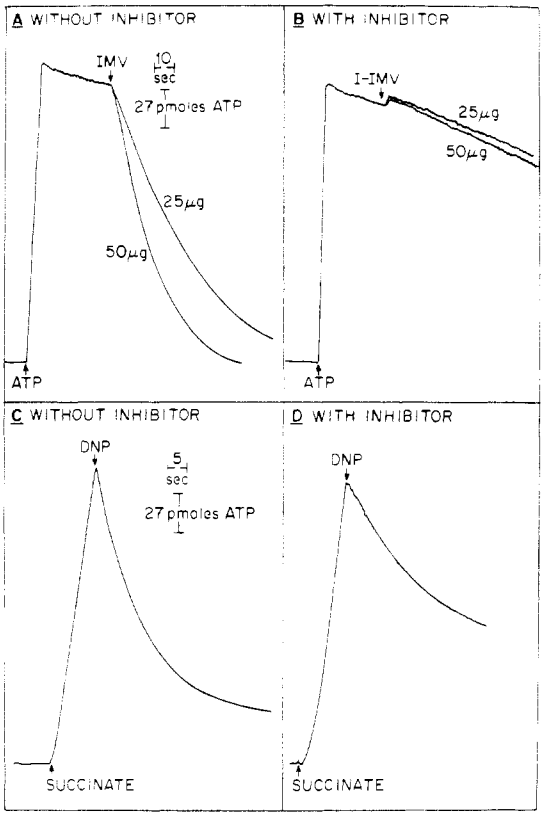

FIGURE 2: Effect of purified rat liver ATPase peptide inhibitor on ATP hydrolytic and synthetic activities of inner membrane vesicles from the same source. (A and B) The reaction medium containing luciferin and luciferase was as described under Methods with indicated amounts of inner membranes vesicles (IMV) or inhibitor-supplemented inner membrane vesicles (I-IMV) prepared as also described under Methods. Where indicated, 2 μL of 0.1 mM ATP was added. (C and D) Inner membrane vesicles (200 μg) were incubated in a total volume of 1 mL for 10 min at room temperature in the basic assay medium described under Methods with 4 μM ADP and without luciferin and luciferase in order to hydrolyze contaminating ATP. Then 10 μL each of 1.5 mM luciferin and 0.2 mg/mL luciferase was added and the light emission recorded in the luminometer. Finally, 10 μL of 100 mM succinate was added to initiate ATP synthesis. Where indicated, 5 μL of 10 mM DNP was added.

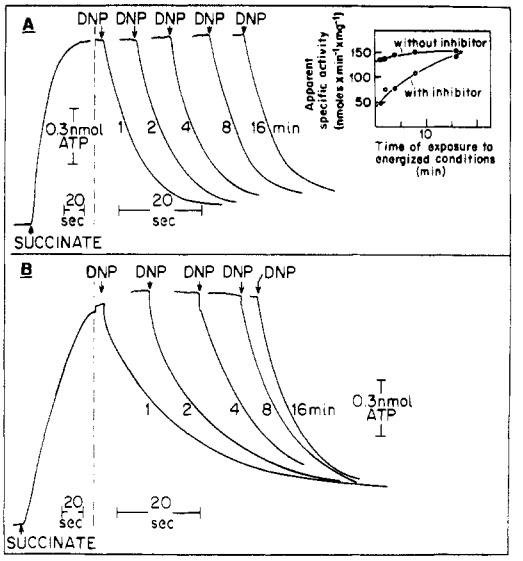

FIGURE 3: Effect of incubation with a respiratory substrate on DNP-induced ATP hydrolysis of inner membrane vesicles in the absence (A) and presence (B) of the ATPase peptide inhibitor. Inner membrane vesicles and inhibitor-supplemented vesicles were prepared as described under Methods. Vesicles (75 μg) were incubated as described in Figure 2C,D except the ADP concentration was 2 μM. ATP synthesis was initiated by addition of 10 μL of 10 mM succinate. Where indicated, ATP hydrolysis was initiated with 5 μL of 10 mM DNP. Inset: Comparison of the effect of time of exposure to energized conditions on the apparent specific ATPase activity of inner membrane vesicles in the absence and presence of ATPase peptide inhibitor. The apparent specific ATPase activity was estimated in each trace shown in (A) and (B) from the decrease of the light response within the first 2 s following addition of DNP.

The inset of Figure 3 depicts more clearly the dependence of ATP hydrolytic capacity of inhibitor-supplemented vesicles on time of exposure to energized conditions. In contrast to vesicles to which no inhibitor peptide was added, and which maintain a constant specific ATPase activity as a function of time exposed to energized conditions, inhibitor-supplemented vesicles have a low specific ATPase activity at short times of exposure which eventually increases to the same specific ATPase activity levels of vesicles free of added inhibitor.

Results presented here show that the ATPase activity of rat liver inner mitochondrial membrane vesicles supplemented with purified ATPase peptide inhibitor is a time-dependent function of the energy state of the membrane.

Capacity of H^+-ATPase To Catalyze ATP Synthesis in the Presence and Absence of the ATPase Peptide Inhibitor: Effect of Energy State. As indicated in Figure 4A, inhibitor-supplemented vesicles, in contrast to control vesicles, exhibit a brief (in the order of seconds) lag phase in the time course of oxidative phosphorylation, provided ATP synthesis is initiated with respiratory substrate, in this case succinate. The lag phase in ATP synthesis was a highly reproducible observation noted in over 25 different experiments. The lag phase in these experiments ranged from 2 to 4 s with an average value of 3 s. (It should be noted that to observe the lag phase in these experiments it was necessary to use a more sensitive time scale than those used in experiments summarized in Figure 1B,C and Figure 2C,D.)

Figure 4A,B shows that the lag phase observed when succinate is added can be prevented if the vesicles are incubated prior to assay with the respiratory substrate NADH. The inset expresses data in Figure 4A,B in such a way as to show the inhibitory effect of the ATPase peptide inhibitor on the initial phase of oxidative phosphorylation. After 2 s of assay, control particles and inhibitor-supplemented particles pretreated with NADH catalyze ATP synthesis at a steady-state rate whereas inhibitor-supplemented particles are more than 70% inhibited. Figure 5 shows that the lag phase (or initial inhibition of ATP synthesis) is also prevented if the assay is initiated with ADP rather than succinate (i.e., the vesicles have already undergone some respiration prior to addition of ADP).

It is clear from these results that exogenously added ATPase peptide inhibitor has a brief inhibitory effect on the rate of oxidative phosphorylation catalyzed by the H^+-ATPase of inner mitochondrial membrane vesicles of rat liver and that this inhibition is dependent on the energy state of the membrane. If the membrane is energized by addition of respiratory substrate prior to initiation of ATP synthesis, no lag phase occurs.

The energized state of the membrane affects only the initial phase of oxidative phosphorylation in inhibitor-supplemented vesicles and is without effect on the steady-state rate of ATP synthesis. This is emphasized in Figure 5 where it is shown that prior incubation of inhibitor-supplemented particles for up to 10 min has no effect on the steady-state rate of ATP synthesis.

Release of the ATPase Peptide Inhibitor from Inhibitor-Supplemented Inner Membrane Vesicles: Effect of Energy State. Data presented above demonstrate that under defined conditions the purified ATPase peptide inhibitor from rat liver

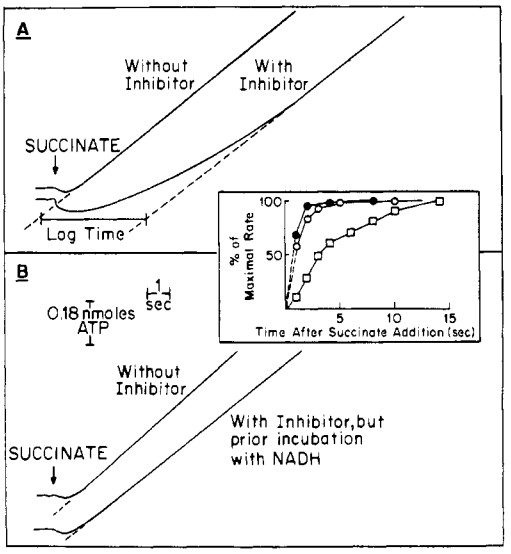

FIGURE 4: Initial events in ATP synthesis of inner membrane vesicles in the absence and presence of ATPase peptide inhibitors. (A) Inner membrane vesicles and inhibitor-supplemented vesicles were prepared as described under Methods. Vesicles (100 μg) were incubated as described in Figure 2C,D except the ADP concentration was 5 μM. ATP synthesis was initiated with 10 μL of 100 mM succinate and monitored as light emission from the luciferin–luciferase couple. (B) Vesicles without inhibitor were prepared and assayed as described in (A). Vesicles (100 μg) with inhibitor were incubated in the assay medium (see Methods) in the presence of 1 mM NADH for 2 min at room temperature, and then 0.4 μg of rotenone was added followed by addition of 5 μM ADP. ATP synthesis was assayed as described in (A). Inset: The rate of ATP synthesis estimated at different time points after addition of succinate is plotted as the percent of the linear steady-state rate (after 15 s). The lag time is defined as the time required to reach 50% of the full activity. (●) Without inhibitor; (○) with inhibitor but prior incubation with NADH; (□) with inhibitor.

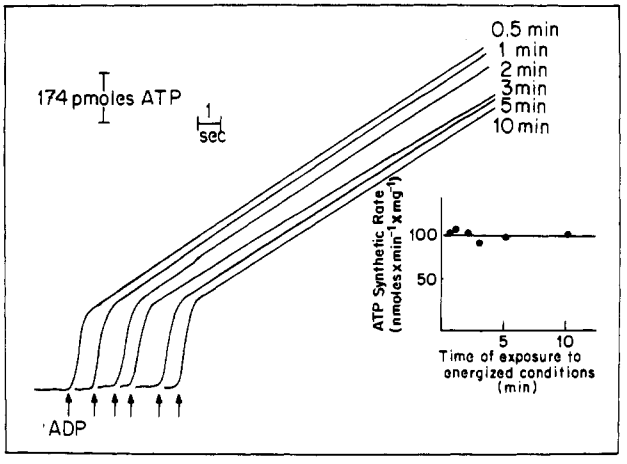

FIGURE 5: Effect of incubation time with a respiratory substrate on the steady-state rate of ATP synthesis in inner membrane vesicles supplemented with ATPase peptide inhibitor. Inhibitor-supplemented vesicles (33 μg) prepared as described under Methods were incubated as indicated in Figure 2C,D except ADP was replaced with 1 mM succinate. Where indicated, ATP synthesis was initiated by addition of 20 μL of 1 mM ADP. The initial burst preceding the steady-state phase represents small amounts of ATP contaminating the ADP.

mitochondria can suppress the initial rates of both ATP hydrolysis and ATP synthesis catalyzed by the H+-ATPase of inner membrane vesicles from the same source. In both cases, the inhibitory effect can be overcome by subjecting the particles to energized conditions prior to assay. Although it might be tempting to suggest that energized conditions induce dissociation of the inhibitor from the enzyme surface, allowing either ATP synthesis or ATP hydrolysis to take place de-

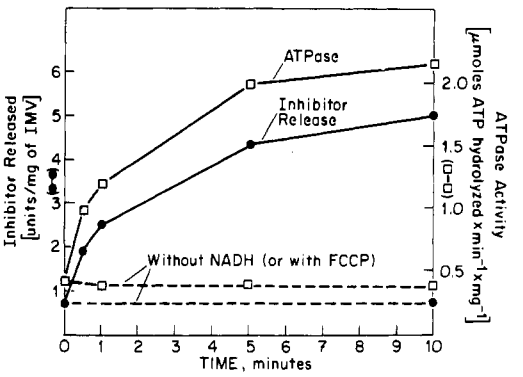

FIGURE 6: Parallel rise in ATP hydrolytic activity accompanying release of the ATPase peptide inhibitor from inhibitor-supplemented vesicles. Inhibitor-supplemented particles (5 mg) were incubated in 12 mL of a medium containing 20 mK K_1–Hepes, 10 mM KP_i, 5 mM $MgCl_2$, 1 mM Na_2EDTA, 20 μM ADP, and 0.25 mg/mL BSA, pH 7.6. The mixture was bubbled with oxygen for about 5 min. Where indicated at time zero on the figure, respiration was initiated with 1 mM NADH. After the indicated time intervals, 2-mL samples were withdrawn and placed into centrifuge tubes containing 2 μL of 1 mM FCCP. The samples were centrifuged at 100000*g* for 30 min at 0–4 °C. The sediment was suspended in H medium (see Methods) and the ATP hydrolytic activity measured in the spectrophotometric assay (see Methods). Cl_3CCOOH (0.1 mL of 100% w/v) was added to 1.9 mL of the supernatant. The precipitate was collected by centrifugation and dissolved in 0.1 mL of 20 mM KP_i, pH 6.8. The ATPase peptide inhibitory activity was measured as described under Methods. (In these experiments, it should be noted that it was crucial to choose a low concentration of vesicles and to keep the concentration of free inhibitor very low, in order to avoid reassociation of the free inhibitor with the vesicles after deenergization. It should be noted also that BSA was included in the incubation mixture to facilitate the recovery of very small amounts of the inhibitor peptide upon Cl_3CCOOH precipitation). Two controls were treated the same way, in one omitting NADH; in the other, FCCP was added before NADH. Both were incubated for 10 min.

pending upon the conditions (energized or nonenergized), the relative time dependencies of the activation of the two catalytic events do not permit such a simple conclusion to be made. Thus, as already emphasized in this report, energized conditions can fully activate ATP synthesis in less than 10 s whereas full activation of ATP hydrolysis requires almost 12 min or 72 times longer. It became essential, therefore, to establish whether or not the ATPase peptide inhibitor is released from inner membrane vesicles as a function of energy state and, if so, whether the time dependence of release correlates with the rapid activation of ATP synthesis or the slow activation of ATP hydrolysis.

Results presented in Figure 6 show that the ATPase peptide inhibitor is released upon energization and depict the time dependence of its release relative to the time dependence of activation of ATP hydrolytic and synthetic activities. In these experiments, inner membrane vesicles were incubated first with inhibitor peptide as described under Methods. The respiratory substrate NADH was added to ascertain whether the ATPase inhibitor peptide could be released by energization. At given time points, the reaction was stopped by addition of the uncoupling agent FCCP. The suspension was centrifuged at high speed and the ATPase activity in the sediment, and the inhibitory activity of the supernatant, determined. The figure shows that energized conditions do release inhibitory activity into the supernatant fraction via a slow process that correlates well with the activation of ATPase activity in the sediment. [Due to the extremely small amounts of protein which are released (less than 1 μg per mg of IMV), only a fraction (~40–60%) could be recovered after Cl_3CCOOH precipitation although BSA was included to improve the recovery.] The

omission of NADH or the addition of FCCP before NADH prevents both the release of the inhibitor peptide and the reactivation of the ATPase.

It is clear from these results that the energy-dependent release of the ATPase inhibitor peptide from inhibitor-supplemented inner membrane vesicles of rat liver is directly related to the capacity of the H^+–ATPase of such vesicles to catalyze ATP hydrolysis rather than ATP synthesis.

Discussion

The major objective of this study was to ascertain whether the energy state of the mitochondrial inner membrane of rat liver alters binding of exogenously added ATPase peptide inhibitor from the same source and whether such an alteration affects the capacity of the H^+–ATPase to catalyze ATPase activity and ATP synthetic activity, or both. This objective has been met, and additional information about the time course of the parameters affected by the energy state has been documented. Energization of the rat liver inner membrane with respiratory substrate has been shown to relieve inhibition of ATP synthesis induced by added inhibitor at a very early time period, in the order of seconds. During this time period, ATP hydrolytic capacity remains markedly suppressed by the ATPase inhibitor peptide, which remains membrane bound. Therefore, activation of ATP synthetic activity is not accompanied by release of the peptide inhibitor but most likely by a weakening of its binding to the enzyme surface. Continued energization of the membrane for several minutes has no further affect on the rate of ATP synthesis which has now reached a steady state. However, the membrane's capacity to catalyze ATPase activity rises. Concomitant with this rise is a release of ATPase peptide inhibitor into the supernatant fraction. Thus, prolonged energization of the inner mitochondrial membrane of rat liver results in a conversion of the H^+–ATPase to an inhibitor-free form.

There have been several studies concerned with the effect of energy state on reactions catalyzed by mitochondrial H^+–ATPase and its relationship to the ATPase peptide inhibitor. Although all of these studies have been carried out with the bovine heart system, rather than the rat liver system, they merit discussion here for comparative purposes. The first study along these lines was carried out by Van de Stadt et al. (1973), who showed that energization of submitochondrial particles containing endogenous inhibitor peptide results in an increase in ATPase activity. In extending these studies, Gómez-Puyou et al. (1979) showed that energization of submitochondrial particles containing inhibitor peptide results in an increase in ATPase activity and eliminates a lag phase in the initial rate of ATP synthesis. Finally, in a concurrent study by Harris et al. (1979), the experiments of Gómez-Puyou et al. (1979) were confirmed; i.e., energization of inhibitor-supplemented submitochondrial particles with respiratory substrate was shown to eliminate the lag phase in the initial rate of ATP synthesis.

Unfortunately, inhibitor release was never monitored in these previous studies on the bovine heart system. Moreover, the relative time course of activation of ATPase and ATP synthetic activities was either not monitored at all (Van De Stadt et al., 1973; Harris et al., 1979) or monitored with different assay systems (Gómez-Puyou et al., 1979). Therefore, it is difficult to critically evaluate the speculative models in the literature which arose out of these earlier studies (Van De Stadt et al., 1973; Ernster et al., 1979). Our results suggest a three-state model (Figure 7) which is supported by experimental data presented in this report on the rat liver system. Incubation of inner membrane vesicles with ATPase peptide inhibitor in

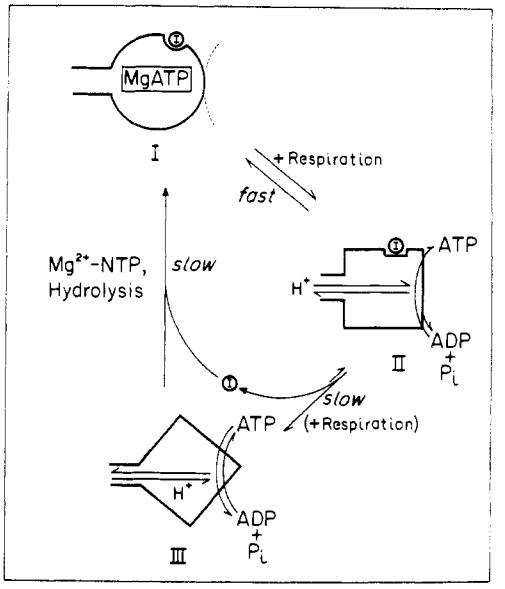

FIGURE 7: Model depicting the dependence of H^+–ATPase–peptide inhibitor interactions on the energy state. (Stage I) In the presence of MgATP and the absence of a membrane potential, the inhibitor peptide is depicted as being bound to the H^+–ATPase, inhibiting both ATP synthesis and ATP hydrolysis. (Stage II) Upon energization, the H^+–ATPase is depicted as undergoing a change in state which reduces the affinity of the inhibitor peptide for its binding site. The H^+–ATPase is now depicted as being catalytically active in synthesizing ATP. It is also considered to be catalytically active in hydrolyzing ATP, but at a slow rate under nonenergized conditions. (Stage III) Reduction in affinity for its binding site at stage II is depicted as causing a slow release of the inhibitor peptide. Inhibitor release is depicted as being accompanied by a slow change in state. The H^+–ATPase is now considered to be fully active in catalyzing either ATP synthesis under the energized conditions shown or NTP hydrolysis if placed under nonenergized conditions in the absence of the inhibitor. If the membrane potential collapses, the H^+–ATPase is depicted as recombining with the inhibitor peptide in the presence of MgATP and forming again the H^+–ATPase complex of stage I.

the presence of MgATP does result in an inhibited state (state I) of the enzyme (Figure 2B,D). Energization of state I particles results in an activation of ATP synthetic activity, consistent with an active state II form of the enzyme containing peptide inhibitor. Prolonged energization is without effect on ATP synthetic activity (Figure 5) but results in inhibitor release with a concomitant rise in the *capacity* of the particles to catalyze ATPase activity under nonenergized conditions (Figures 3 and 6), consistent with an active state III or inhibitor-free form of the enzyme.

Since the original study of Pullman & Monroy (1963), many investigators have suggested that ATPase peptide inhibitors may be physiological regulators of H^+–ATPases [for a review, see Pedersen et al. (1981)]. Results presented here are not at all inconsistent with this view and, in fact, could be taken as providing additional support for it. Clearly, the primary effect of this class of peptides is, as their name implies, to suppress ATPase activity catalyzed by H^+–ATPases. Significantly, they do not alter the steady-state rate of ATP synthesis probably because the rate-limiting step of oxidative phosphorylation is the oxidation of substrates (Sorgato et al., 1980). It seems of special interest that the rat liver inhibitor studied here does not dissociate from inner membrane vesicles during short energization pulses which induce ATP synthesis. Rather, it remains bound and "ready" to suppress the reverse reaction as soon as an energization pulse is terminated. If the same type of phenomenon is found to occur in intact mitochondria, this would suggest that a major role of the peptide inhibitor is to preserve newly synthesized ATP following a

burst of "phosphorylating" respiration.

Finally, it should be kept in mind that rat liver mitochondria can readily catalyze ATP-dependent functions like Ca^{2+} uptake, transhydrogenation from NADH to NADP, and reduction of NAD^+ by reverse electron flow. However, it is not clear whether the H^+-ATPase works "functionally" in the ATP hydrolytic direction in rat liver mitochondria under physiological conditions. If it does, it would seem that additional regulatory factors may come into play in certain physiological situations to prevent the H^+-ATPase from recombining with its peptide inhibitor.

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