MITOCHONDRIAL ATPase ACTIVITY AND AdN TRANSLOCATION WITH ε-ATP AS SUBSTRATE

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1. Introduction

Rat liver mitochondria display two types of ATPase activities: a low level Mg-stimulated ATPase activity which appears to be located within the intermembrane space and is not directly associated with oxidative phosphorylation [1–4] and a DNP-stimulated* F_1-ATPase activity that is located on the inner surface of the inner membrane and is associated with the energy conservation process [4–8]. While the Mg-stimulated ATPase activity has not been well characterized, the F_1-ATPase that is associated with oxidative phosphorylation has been studied extensively [6,8]. Prior to interaction with the latter enzyme, AdN added to a mitochondrial suspension must first traverse the outer mitochondrial membrane and intermembrane space and then be translocated into and possibly through the inner membrane by the ATP/ADP facilitative exchange carrier [4,9–11]. This AdN translocase maintains a strict specificity for an intact adenine base and at least two phosphate groups [12]. Thus, inosine, quanosine, and uridine nucleotides are not exchanged to appreciable extents [4,11]. However, it has been shown that the 1-*N*-oxide analog of ATP can be translocated approximately 30% as well as ATP [13].

ε-ATP (1,N^6-ethenoadenosine 5′-triphosphate), a fluorescent analog of ATP [14], has been found to be a good replacement for ATP as a substrate for several soluble enzymes, but not for the chloroplast affiliated ATPase [15]. We have investigated the interaction of ε-ATP with mitochondrial ATPase and the AdN carrier and find that this analog behaves as a capable substrate. Its extent of utilization relative to ATP varies significantly with the concentration of added AdN. The ε-ATP hydrolytic activity has been demonstrated as atractyloside-sensitive, oligomycin-sensitive, but DNP-insensitive.

2. Materials and methods

Liver mitochondria were isolated from male Long–Evans rats essentially according to the method of Kielley and Kielley [16], using 0.25 M sucrose–0.001 M EDTA as the isolation medium. Mitochondrial protein concentration was determined by the biuret procedure [17] modified as follows. The final 5.0 ml volume contained 0.4% (w/v) Na-deoxycholate, and a 3:2 combining proportion of Biuret reagent (Sigma) with respect to the other constituents present. Human albumin, gamma globulin, and bovine serum albumin were used as standards. Orthophosphate concentration was determined by a modified Fiske and Subbarow method [18] consisting of the following final reagent concentrations: 0.006% (w/v) 1,2,4-aminonaphthol sulfonic acid, 0.025% (w/v) sodium sulfite, 0.73% (w/v) sodium bisulfite, and 0.208% (w/v) ammonium molybdate in 0.416 N sulfuric acid. These reagents were obtained from Sigma.

Tightness of coupling of isolated mitochondria prior to and following the ATPase studies was routinely evaluated by methods similar to those described previously [19]. The AdN hydrolysis incubations were run at 28°C and contained 5 mM

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Abbreviations: AdN = adenine nucleotide; ε-ATP, 1,N^6-ethenoadenosine 5′-triphosphate; DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetraacetic acid.

$MgCl_2$, 100 mM sucrose, 20 mM Tris-maleate buffer, pH 7.2, various concentrations of AdN, and an average of 1.37 mg mitochondrial protein/ml of incubation. Other additions are reported with the appropriate tables. Controls for any endogenous orthophosphate in the mitochondrial aliquots, as well as that due to non-enzymatic hydrolysis of AdN were routinely performed. ATPase reactions were triggered by the addition of mitochondria. However, during the atractyloside (potassium salt, Sigma) incubations, the mitochondria were allowed to preincubate with the inhibitor for 2 min prior to initiating the reaction by addition of AdN. Reactions were terminated after 18 minutes with 6% (w/v) perchloric acid, centrifuged, and the supernatants were assayed for orthophosphate as described above.

ϵ-ATP was synthesized according to a procedure derived from Secrist et al. [14]. Chloroacetaldehyde (Pfaltz and Bauer Inc.) was distilled under reduced pressure at 36–37°C for 45 min. 26 ml of distilled chloroacetaldehyde were added to 2.5 mmol of disodium ATP (Sigma) and the pH of the resulting solution was adjusted to 4.3. The reaction was run at a constant pH of 4.3 and at room temperature in a pH stat (Radiometer pH Stat Titrator type TTTlc and Titrigraph type SBR2) for 18–24 h, with sodium bicarbonate as titrant. The progress of the reaction was followed by thin layer chromatography (Eastman Chromatogram, Cellulose) employing isobutyrate–H_2O–NH_4OH (66:32:2) as the running solvent and the ϵ-ATP spot was visualized under u.v. illumination. ϵ-ATP was extracted essentially as indicated by Secrist et al, and stored at −80°C.

The purity of ϵ-ATP with respect to non-reacted AdN was assayed chromatographically and spectrophotometrically. TLC showed no evidence of unreacted AdN contamination. U.v. absorbances were obtained at several λ_{max} values (265 nm and 275 nm) using a Varian Model 635 spectrophotometer. Purity of the fluorescent product was approx. 98%.

3. Results and discussion

The substrate capability of ϵ-ATP has been measured by orthophosphate release, and is a function of the sequential interaction of the AdN with the Mg-stimulated ATPase, the AdN carrier, and the DNA-sensitive ATPase. Table 1 demonstrates the utilization of ϵ-ATP relative to ATP as a function of AdN concentration, and shows that ϵ-ATP behaves as a reasonably good substrate for total latent ATPase activity in coupled mitochondria. It is noteworthy that as the AdN concentration was increased from 1 to 10 mM, the relative substrate efficiency of ϵ-ATP decreased 26%. Most of this drop in efficiency occurred between 3 and 10 mM substrate levels. ϵ-ATPase activity appears to be maximal in this system at analog concentrations near 3 mM whereas ATP hydrolytic activity continues to rise at substrate concentrations between 3 and 10 mM.

2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, is known to stimulate the activity of the ATPase enzyme that is directly associated with energy conservation [5,7]. The results presented in table 2 indicate that regardless of the AdN con-

Table 1
Total ATPase and ϵ-ATPase activity: the effect of AdN concentration

Substrate (mM)	P_i released ($\times 10^7$ moles P_i/mg/18 min)	Relative substrate effectiveness (%)
ATP 1.0	6.35 (3)	100
ATP 3.0	12.93 (3)	100
ATP 10.0	15.45 (6)	100
ϵ-ATP 1.0	4.35 (3)	69
ϵ-ATP 3.0	7.88 (3)	61
ϵ-ATP 10.0	6.72 (6)	43

Experimental procedure as described in Materials and methods. Values in parentheses represent the number of repetitions for a given incubation. Data are corrected for non-enzymic hydrolysis of AdN and endogenous mitochondrial P_i.

Table 2
The Sensitivity of ATPase and ϵ-ATPase activity to 2,4-DNP

ATP or ϵ-ATP (mM)	P_i released (X 10^7 moles P_i/mg/18 min)		Stimulation (%)	
	ATP	ϵ-ATP	ATPase	ϵ-ATPase
1.0	6.35	4.35	–	–
1.0 + DNP (50 μM)	10.04	4.34	58	0
3.0	12.93	7.88	–	–
3.0 + DNP (50 μM)	27.60	8.66	113	10
10.0	16.58	7.50	–	–
10.0 + DNP (50 μM)	51.25	8.73	209	16
10.0	16.37	5.94	–	–
10.0 + DNP (200 μM)	68.48	6.72	318	13

Conditions as described in table 1. Data represent means of incubations run an average of 3 times.

centration employed, those levels of uncoupler which greatly stimulated the hydrolysis of ATP, manifested little enhancement in the hydrolysis of ϵ-ATP. Utilizing 1 or 3 mM ATP, 50 μM DNP elicited a 58% and 113% enhancement of ATPase activity, respectively. However, employing 1 mM ϵ-ATP, 50 μM DNP failed to stimulate ϵ-ATPase activity, and caused only a 10% enhancement when the analog concentration was increased to 3 mM. In the presence of 10 mM substrate, the addition of 50 μM uncoupler increased ATP hydrolysis by more than 200% but ϵ-ATP hydrolysis by only 16%. Qualitatively similar results were obtained with a higher uncoupler concentration (200 μM). Since ϵ-ATPase activity could not be significantly stimulated by uncoupler addition, the ability of this analog to act as a substrate for the oxidative phosphorylation enzyme seemed questionable. It was thought that perhaps the intermembrane Mg-stimulated, DNP-insensitive ATPase activity that is not directly associated with the energy coupling process might be totally responsible for the orthophosphate release from ϵ-ATP.

To determine the ability of the AdN carrier and the oxidative phosphorylation ATPase to utilize ϵ-ATP as a substrate, the action of atractyloside was examined, since the latter is known to be a specific inhibitor of the translocase [9,11]. It has been observed that in the presence of atractyloside and exogenous ATP, the inner membrane DNP-sensitive F_1-ATPase activity is markedly inhibited [1,20] while the low level intermembrane Mg-stimulated ATPase activity remains unchanged [2,3]. Therefore, an atractyloside-induced inhibition of orthophosphate release from ϵ-ATP would indicate that this fluorescent

Table 3
Atractyloside inhibition of adenine nucleoside triphosphate hydrolysis

Substrate (mM)	Atractyloside Concentration (mM)	P_i released (X 10^7 moles P_i/mg/18 min)	Inhibition (%)
ATP 1.0 (control)	0	6.35 (3)	–
ATP 1.0	3.3	3.79 (3)	40
ϵ-ATP 1.0 (control)	0	4.35 (3)	–
ϵ-ATP 1.0	3.3	2.74 (3)	37

Conditions as described for table 1, except the mitochondria were preincubated with atractyloside for 2 min prior to initiation of the reaction by AdN addition.

Table 4
Oligomycin inhibition of ATPase and ATPase activity

Substrate (mM)	Oligomycin (μg/ml)	P_i released (× 10^7 moles P_i/mg/18 min)	Inhibition (%)
ATP 3.0 (control)	0	11.38 (6)	–
ATP 3.0	0.4	6.20 (7)	46
ϵ-ATP 3.0 (control)	0	6.26 (7)	–
ϵ-ATP 3.0	0.4	4.30 (8)	31
ATP 10.0 (control)	0	17.02 (6)	–
ATP 10.0	0.4	8.98 (9)	47
ϵ-ATP 10.0 (control)	0	5.76 (4)	–
ϵ-ATP 10.0	0.4	2.61 (6)	55
ATP 10.0 (control)	0	16.08 (4)	–
ATP 10.0	4.0	2.77 (5)	83
ϵ-ATP 10.0 (control)	0	4.57 (3)	–
ϵ-ATP 10.0	4.0	1.74 (5)	62

Oligomycin was made up in DMSO and controls contained appropriate DMSO. 0.4 μg/ml oligomycin ≈ 10^{-6} M. All other conditions as described for table 1.

analog was, in fact, capable of facilitative transport and subsequent utilization by the DNP-sensitive ATPase. Table 3 indicates that with 1 mM ATP or ϵ-ATP, the presence of 3.3 mM atractyloside resulted in a 40% and 37% inhibition of AdN hydrolysis, respectively. Therefore it is concluded that approximately 40% of either substrate can be translocated and subsequently hydrolyzed by the oxidative phosphorylation F_1-ATPase. The residual hydrolysis which persists in the presence of atractyloside is probably due to the activity of the intermembrane, Mg-stimulated ATPase. The magnitude of this latter enzymic activity coincides with the observations of other workers [1–3].

Unlike atractyloside, oligomycin is a potent inhibitor of both the F_1-ATPase and the Mg-stimulated ATPase activities [1,3,21]. Table 4 demonstrates that low concentrations of oligomycin (0.4 μg/ml), in the presence of either 3 or 10 mM ATP causes approximately a 50% inhibition of hydrolysis. Under similar conditions the inhibition of ϵ-ATP hydrolysis appears to be dependent on substrate concentration, for at ϵ-ATP concentrations of 3 and 10 mM we found inhibitions of 31% and 55% respectively. With higher oligomycin levels (4 μg/ml) and 10 mM AdN, the extents of hydrolytic inhibition for ATP and ϵ-ATP rose to 83% and 62%, respectively. The fact that oligomycin inhibition of total ATPase is greater than that produced with atractyloside, constitutes further evidence that ATP and ϵ-ATP act as efficient substrates for both of the mitochondrially associated ATPase activities. These data also support the proposal that ϵ-ATP can be translocated via the AdN carrier. Therefore, it appears that under carefully stipulated conditions, ϵ-ATP may be useful in elucidating the recently described functional relationship between the AdN carrier and the F_1-ATPase [22–24], possibly by utilizing the molecule's fluorescent properties. In an attempt to elucidate the interaction between ϵ-ATP and the various components involved in oxidative phosphorylation, further investigations are underway employing both whole mitochondria as well as submitochondrial particles produced via sonication, and will be reported elsewhere.

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