PARTICIPATION OF εADP AND εATP IN THE REACTIONS OF OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

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SUMMARY - The 1,N^6-ethenoadenine nucleotide analogs εADP and εATP, contrary to recent findings (1), are shown to be unable to penetrate the inner mitochondrial membrane of intact rat liver mitochondria and can not be used as substrates by the respiratory chain enzymes in oxidative phosphorylation. On the other hand, these analogs are able to participate in transphosphorylation reactions, being good substrates for mitochondrial phosphotransferases located in the intermembrane space, such as nucleosidediphosphate kinase and adenylate kinase.

INTRODUCTION - Recent advances in nucleotide chemistry have led to the availability of an ever increasing number of synthetic adenine nucleotide analogs (2) of which many have been used to investigate the yet not fully understood chemistry of oxidative phosphorylation (3-9). Among these analogs, εADP and εATP occupy a special place due to their remarkable fluorescent properties, which allow an excitation outside the normal absorption range of proteins and nucleic acids (10), while at the same time, the structural modification on the purine ring is tolerated by a large number of enzymes (2).

Very recently Kaplan and Coleman (1) investigated the interaction of εATP with mitochondrial ATPase and with the adenine nucleotide carrier, concluding that this analog is a good substrate and that it does participate in the reactions of oxidative phosphorylation. However, we had found previously that the adenine N^1-oxide nucleotides,

Abbreviations: εADP, εATP, 1,N^6-ethenoadenosine di- and triphosphate; o^{1}ADP, o^{1}ATP, adenosine N^1-oxide di- and triphosphate; AMP-PNP, adenylyl (β,γ-imido)-diphosphate; DNP, 2,4-dinitrophenol.

which have very similar structural and enzymatic properties to the ε-adenine nucleotides, are not translocated across the inner mitochondrial membrane, i.e. they do not participate in the reactions of oxidative phosphorylation (11,12,9). Since 1,N^6-ethenoadenosine derivatives are now commercially available and widely used due to their unique fluorescent properties, we considered it important to reinvestigate these analogs and clarify the question whether they can be used by the respiratory chain enzymes.

MATERIALS AND METHODS - ADP, ATP, AMP-PNP, NADH, phosphoenolpyruvate, lactate dehydrogenase (EC. 1.1.1.28), glucose-6-phosphate dehydrogenase (EC. 1.1.1.49), pyruvate kinase (EC. 2.7.1.40), phosphofructokinase (EC. 2.7.1.11) and hexokinase (EC. 2.7.1.1) were commercial products from Boehringer Mannheim. εADP and εATP, prepared according to Secrist *et al.* (9), were a gift of Prof. M. Klingenberg, Munich. o^1ATP and o^1ADP were prepared according to previously published procedures (9). The purity of each of the nucleotides was checked by their spectroscopic properties and by thin layer chromatography. For the evaluation of the nucleotide concentrations the following millimolar extinction coefficients were used: 15.4 at 260 nm for ADP, ATP and AMP-PNP; 40.8 at 233 nm for o^1ADP and o^1ATP; 5.7 at 265 nm for εADP and εATP. All investigated di- and triphosphate nucleotides (except AMP-PNP) are substrates for pyruvate kinase and phosphofructokinase respectively, so that their concentration could also be determined enzymatically in the presence of excess enzyme and phosphoenolpyruvate or fructose-6-phosphate, respectively. There was good agreement (±3%) between these two methods. "Lubrol-particles" were obtained from 3 ml of a mitochondrial suspension in 250 mM sucrose containing about 120 mg of protein which were treated with 3 ml of lubrol WX_o in 250 mM sucrose (20 mg/ml) and stirred gentle for 15 minutes at 2^o. Afterwards 6 ml of 250 mM sucrose were added and the mixture centrifuged at 10,000 g for 15 minutes, the supernatant collected and again centrifuged at 150000 g for 1 hour. The sediment, containing about 25 mg of protein, was used directly under the name of "lubrol-particles", which were able to oxidize succinate at a rate of 60-70 ng Atoms/min/mg protein, with a P/O ratio of 0.4-0.45.

For the isolation of rat liver mitochondria, the protein determination, measurement of the mitochondrial respiration and oxidative phosphorylation, the exchange of intramitochondrial ^{14}C-labelled adenine nucleotides with externally added nucleotides, the determination of apparent formation constants with Mg^{2+}, and the assay of ATPase activity, we used methods described in previous publications (9, 11,12).

RESULTS AND DISCUSSION - Addition of εADP to respiring intact rat liver mitochondria has no effect on respiration but subsequent addition of ADP stimulates the respiration, which returns to state 4 much later than expected from the added amount of ADP. A new addition of

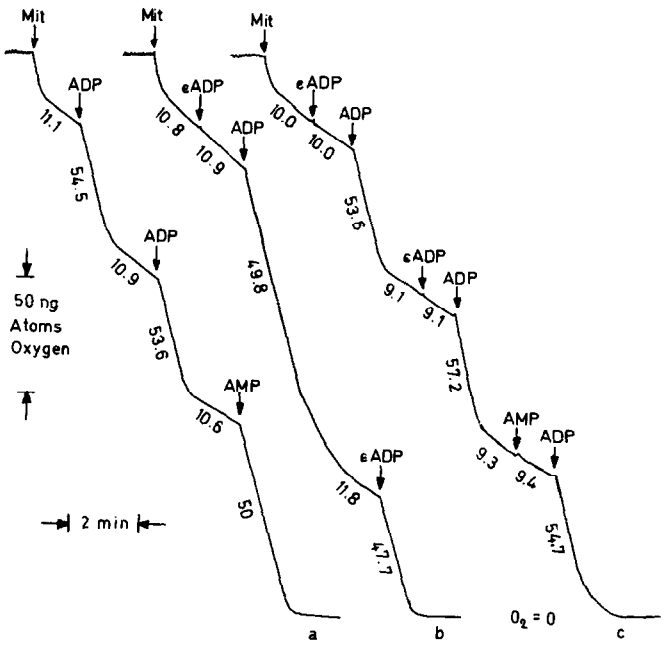

Figure 1. Effect of εADP on the Respiratory Rate of Rat Liver Mitochondria.

The basic respiratory medium contained at 0.5 ml final volume and 24°: 180 mM sucrose, 50 mM KCl, 25 mM Tris-HCl (pH 7.4), 5 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 1 mg bovine serum albumin, 1 mM EDTA and 5 mM glutamate. For each experiment 1.1 mg of mitochondrial protein was used. Additions were as follows: trace a, 0.23 mM ADP, 0.23 ADP and 0.25 mM AMP; trace b, 0.28 mM εADP, 0.23 mM ADP and 0.28 mM εADP; trace c, 0.28 mM εADP, 0.23 mM ADP, 0.28 mM εADP, 0.23 mM ADP, 0.25 mM AMP and 0.23 mM ADP; no Mg^{2+} was present in the experiment in trace c.The numbers beside the traces indicate the oxygen consumption as ng-atoms per min per mg of protein.

εADP now stimulates the respiration (Fig. 1, trace b). The ratio (εADP+ADP)/O yields a value of 2.6 with glutamate as substrate, which is very close to the reference value obtained with ADP alone (trace a). In the absence of exogenous Mg^{2+}, εADP and AMP show no effect at all on the mitochondrial respiration (trace c). These experiments lead to the conclusion that the stimulation of mitochondrial respiration through addition of εADP to mitochondria after the state 3 to state 4 transition, is due to the coupling of the respiratory chain linked

Table 1. Formation Constants (K_f) for Adenine-, Adenine N^1-oxide- and 1,N^6-ethenoadenine Nucleotides with Mg^{2+}

Nucleotide		K_f (M^{-1})
εADP	(3)	1,530 ± 80
ADP	(3)	1,600 ± 100
o¹ADP	(4)	1,650 ± 40
εATP	(2)	9,800 ± 360
ATP	(3)	10,200 ± 350
o¹ATP	(4)	10,900 ± 420

Measurements were made at pH 8.0 and 24° at a final volume of 10 ml in a medium containing 50 mM KCl, 50 mM Tris-HCl, 0.05 to 0.3 mM nucleotide, 0.2 mM $MgCl_2$ and 50 mg Dowex 1X4. The number of experiments is given in parentheses.

ADP phosphorylation on the inner side of the inner mitochondrial membrane, to the reaction catalysed by nucleosidediphosphate kinase on the outer side of the inner mitochondrial membrane. In the absence of exogenous Mg^{2+}, the phosphorylation of ADP is not affected (the matrix contains sufficient Mg^{2+} to support the phosphorylation of ADP through the respiratory chain enzymes), but the transphosphorylation of εADP through the nucleosidediphosphate kinase reaction is blocked.

To explain the lack of εADP phosphorylation by the respiratory chain enzymes, the following possibilities must be considered:

1. εADP and/or εATP do not bind Mg^{2+} and can not yield an active nucleotide-metal ion complex.
2. εADP is not translocated across the inner mitochondrial membrane.
3. εADP is no substrate for the enzymes of oxidative phosphorylation.

As shown in Table 1 there are no significant differences in the Mg^{2+} binding between the natural and the modified adenine nucleotides. As expected (13), the nature of the base does not strongly affect the formation constants with Mg^{2+}. Table 2 shows that the exchange

Table 2. Exchange of Intramitochondrial ^{14}C-labelled ATP with Extramitochondrial ATP-analogs

Additions	^{14}C-ATP Exchanged (%)
ATP	100
ATP+atractyloside	6.1
AMP-PNP	27.4
AMP-PNP+atractyloside	4.1
εATP	<1
εATP+atractyloside	<1

Mitochondria (4.4 mg of protein) which had been previously loaded with ^{14}C-ATP, were incubated for 10 min at 2° in 110 mM KCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA at a final volume of 0.4 ml. The reaction was triggered by addition of 0.06 mM nucleotide. Atractyloside when present, was 0.05 mM. The incubation was stopped by centrifugation and 0.2 ml of the supernatant used for liquid scintillation counting.

between internal ^{14}C-ATP and externally added εADP or εATP is negligible compared to the exchange of ATP or AMP-PNP. Both ATP and AMP-PNP exchanges are atractyloside-sensitive as shown by Klingenberg *et al.* (14). According to these results, an exchange with the intramitochondrial adenine nucleotide pool must be excluded, although it is possible that εADP and/or εATP binds to the adenine nucleotide translocase in either a specific or nonspecific way.

Since Kaplan and Coleman's conclusion that εATP is translocated across the inner mitochondrial membrane was based on the atractyloside sensitivity of Mg^{2+}-stimulated ATP hydrolysis (1), we reexamined this effect with intact mitochondria, with osmotically shocked mitochondria and with lubrol-particles, which in many respects resemble sonicated mitochondria (15). As shown in Table 3, DNP greatly stimulates the ATP hydrolysis in intact mitochondria, but is practically without effect on the hydrolysis of εATP. 0.05 mM atractyloside reduces the ATPase activity below the "basal level", both in the presence and absence of DNP, regardless whether ATP or εATP are used as substrates. With lubrol-particles, the hydrolysis of ATP and εATP

Table 3. Hydrolysis of ATP and εATP by Different Rat Liver Mitochondrial Preparations

Additions	Intact mitochondria		Osmotically shocked mitochondria*		Lubrol-particles	
	ATP	εATP	ATP	εATP	ATP	εATP
None	33	37	151	144	687	644
DNP	295	40	257	136	698	680
Atractyloside	18	24	98	89	654	642
Oligomycin	11	15	18	22	32	19
DNP+ atractyloside	20	25	101	83		
DNP+oligomycin	16	17	22	25		

*After the second washing the mitochondrial pellet was resuspended in distilled water at a protein concentration of 5 mg/ml.

The test medium contained at 0.2 ml final volume: 100 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 2 mM $MgCl_2$, 5 mM PEP, 2U pyruvate kinase and 0.05 to 0.1 mg protein of mitochondrial preparations. DNP was 100 µM, atractyloside 50 µM and oligomycin 2µg. The reaction was started with ATP or εATP (2 mM final concentration), and incubated for 10 minutes at 30° (24° for lubrol particles). It was terminated by addition of 0.1 ml 20% trichloroacetic acid and the inorganic phosphate determined in the protein free filtrate (20). The ATPase activity, a mean value of three separate experiments, is expressed in nmoles P_i released/min/mg protein. (PEP=phosphoenolpyruvate.)

is almost identical and shows no activation by DNP or inhibition by atractyloside. Oligomycin, inhibits the ATPase activity with intact and osmotically shocked mitochondria, and also with lubrol-particles, with either ATP or εATP as substrate.

In order to establish whether the permeability barrier of intact mitochondria alone is responsible for the fact that εADP does not participate in the phosphorylation reactions of the respiratory chain, we investigated the oxidative phosphorylation of ADP, εADP and o¹ADP by lubrol-particles. As shown in Table 4, in the presence of succinate, natural ADP is phosphorylated at a normal rate (15), while 20 µM DNP inhibit the oxidative phosphorylation by over 80%. Neither o¹ADP nor εADP can act as substrate in the oxidative phosphorylation catalysed by these lubrol-particles, and if added in a 5 fold excess

Table 4. Participation of ADP, εADP and o¹ADP in Oxidative Phosphorylation Catalysed by Rat Liver Lubrol-Particles.

Additions	nmoles NADPH formed/min/mg protein
200 μM ADP	31
40 μM ADP	23
40 μM ADP+200 μM o¹ADP	17
40 μM ADP+200 μM εADP	16
40 μM ADP+20 μM DNP	4
200 μM o¹ADP	0
200 μM εADP	0

The reaction medium contained at 24° per 1 ml, 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 5 mM potassium phosphate (pH 7.4), 2.5 mM $MgCl_2$, 2 mg defatted bovine serum albumin, 0.5 mM EDTA, 5 mM glucose, 0.4 mM NADP, 5U hexokinase, 1.8U glucose-6-phosphate dehydrogenase, 0.28 mM AMP and 0.34 mg lubrol-particles. The submitochondrial particles were incubated 3 min prior to the initiation of the reaction by addition of 5 mM succinate and nucleotide. The absorption increase at 366 nm corresponding to the reduction of NADP was measured with an Eppendorf photometer, equipped with a W+W type 4410 recorder (full scale deflection 0.25 absorbance units).

to the natural nucleotide, the phosphorylation rate of ADP drops by 30% or 26%, respectively.

CONCLUSIONS - The Mg^{2+}-dependent ATPase activity which in intact mitochondria is very small compared to the DNP-stimulated activity (16-18), is independent of the nature of the nucleotide. This applies not only to εATP, but also to such analogs as o¹ATP (11), ITP and 8-BrATP (Bârzu *et al.*, unpublished). Partial damage of the mitochondria, such as through osmotic shock, increases the activity of the substrate-unspecific ATPase and decreases the sensitivity for atractyloside inhibition. Taking into consideration that mitochondrial preparations often contain 5-20% damaged mitochondria, which behave similarly to those shocked osmotically, the ATPase activity could reflect not so much the effect of the investigated nucleotide analogs as the quality of the mitochondrial preparations (1). Care should be also taken with high analog concentrations (10 mM) or high atracty-

loside concentrations (3.3 mM), since a leakage of these compounds into the inner mitochondrial compartment might become responsible for the effect of MgATPase inhibition (19); such effects then could be missinterpreted as a specific transport of nucleotides into mitochondria.

Concluding this investigation we can say that, at least with rat liver mitochondrial preparations, the 1,N^6-ethenoadenine nucleotides, like the adenine N^1-oxide nucleotides, are unable to penetrate the inner mitochondrial membrane and can not be used as phosphate acceptors in oxidative phosphorylation. Recent data obtained in our laboratory on the nucleotide specificity of beef heart submitochondrial particles, strongly support this conclusion.

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