NET UPTAKE OF ADENINE NUCLEOTIDES IN ISOLATED RAT LIVER MITOCHONDRIA

Gregory K. ASIMAKIS* and June R. APRILLE
Department of Biology, Tufts University, Medford, MA 02155, USA

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1. Introduction

The adenine nucleotide content of rat liver mitochondria increases during perinatal development [1–3]. Since the total adenine nucleotides (ATP + ADP + AMP) of the whole liver remain constant during the perinatal period [2], a shifting of the adenine nucleotide pool from the cytosolic to the mitochondrial compartment is inferred. In adult rats glucagon injection has been reported to induce a 60–65% increase in the total adenine nucleotide content of liver mitochondria [4,5]. We reported the net uptake of adenine nucleotides in liver mitochondria that had been depleted of endogenous adenine nucleotides in vitro [6]. The objective of this was to examine further a possible mechanism by which mitochondria can accumulate adenine nucleotides from the extramitochondrial space.

2. Materials and methods

2.1. *Preparation of mitochondria*

Mitochondria were isolated from livers of adult male Charles River CD-1 rats as in [6]. Briefly, the tissue was homogenized in 250 mM sucrose, 1 mM EDTA, and 1 mM Tris–HCl (pH 7.4) (20%, w/v) and centrifuged at 600 × *g* for 10 min. The supernatant was centrifuged at 8000 × *g* for 10 min. Unless indicated differently, the mitochondrial pellet was washed once in the sucrose–Tris–EDTA medium and once in 250 mM sucrose and 1 mM Tris–HCl (pH 7.4). The washed mitochondria were suspended in sucrose–Tris to 30–40 mg protein/ml. All protein concentrations were determined by a Biuret procedure [7].

2.2. *Adenine nucleotide depletion*

Mitochondria (1.5 mg/ml) were incubated for 5 min at 30°C with 1 mM tetrapotassium pyrophosphate in 225 mM sucrose, 75 mM KCl and 5 mM Tris–HCl (pH 7.4). At the end of the incubation time, the suspension was chilled and centrifuged at 8000 × *g* for 10 min. The pellet was then suspended in sucrose–Tris to ~10 mg protein/ml. The mitochondria treated in this manner are referred to as depleted or pyrophosphate-treated mitochondria.

2.3. *Adenine nucleotide loading*

Mitochondria (~1.2 mg/ml) were incubated at 30°C with adenine nucleotides (ATP, ADP or AMP) in 225 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM K_2HPO_4–KH_2PO_4, 5 mM $MgCl_2$ and 10 mM Tris–HCl (pH 7.4). The incubation and assay conditions are given in the figure legends.

2.4. *Extraction and assay of mitochondrial adenine nucleotides*

Mitochondrial protein (8–10 mg) was suspended in 10 ml cold sucrose–Tris, then centrifuged at 8000 × *g* for 10 min. The pellet was extracted in 6% perchloric acid for the determination of ATP, ADP and AMP [6].

3. Results

Pyrophosphate will deplete isolated liver mitochondria of endogenous adenine nucleotides, and the depletion is inhibited by atractyloside [6]. The depletion appears to occur as an exchange of external pyro-

* To whom reprint requests should be directed at present address: Division of Biochemistry, University of Texas Medical Branch, Galveston, TX 77550, USA

phosphate for internal adenine nucleotides over the adenine carrier [6]. In an attempt to reverse this process, the pyrophosphate-treated mitochondria were incubated with ATP, ADP, or AMP. In the experiment shown in fig.1, the total adenine nucleotide content of the pyrophosphate-treated mitochondria was <1 nmol/mg protein (the adenine nucleotide content of freshly prepared mitochondria was 10–12 nmol/mg protein). Incubation of the depleted mitochondria for 10 min with ATP resulted in accumulation of mitochondrial adenine nucleotides to ~80% of the normal level. Adenine nucleotide accumulation was less with ADP as the external source of adenine nucleotides. Even less accumulation was noted when the depleted mitochondria were incubated with AMP. When adenine nucleotides were not added during the reloading step, an additional decrease in adenine nucleotides was noted (not shown). As indicated in fig.1, atractyloside did not inhibit adenine nucleotide accumulation.

Incubation of mitochondria with pyrophosphate did not result in accumulation of intramitochondrial pyrophosphate unless NaF was present to inhibit mitochondrial pyrophosphatase activity [6]. The extent of adenine nucleotide accumulation was not enhanced when the experiment shown in fig.1 was repeated in the presence of NaF to insure that the depleted mitochondria were loaded with PP_i. These results indicate that the accumulation of adenine nucleotides did not occur via the adenine carrier, and did not occur in exchange for intramitochondrial pyrophosphate.

Table 1 shows the effects of various mitochondrial

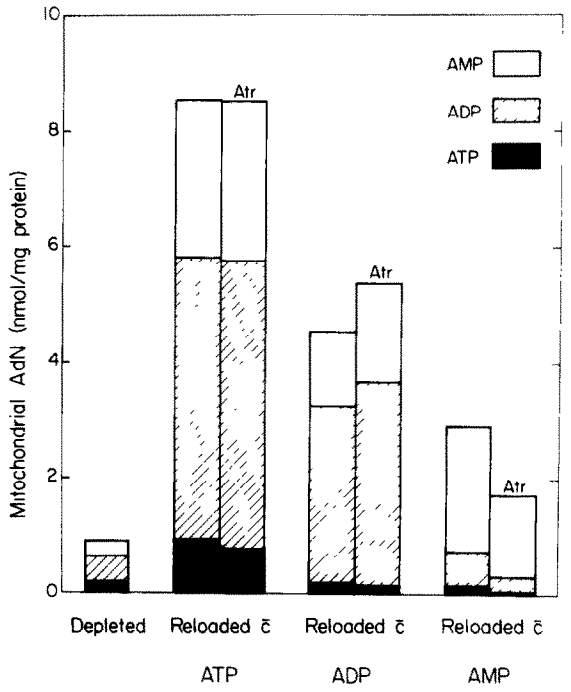

Fig.1. Adenine nucleotide reloading in pyrophosphate-treated mitochondria. Mitochondria were depleted of adenine nucleotides by treatment with pyrophosphate. The depleted mitochondria were then incubated with 1 mM ATP, 1 mM ADP or 1 mM AMP for 10 min in a pyrophosphate-free medium. Where indicated, atractyloside (Atr) was added to 50 μM final conc. The mitochondria were then washed in sucrose–Tris and extracted for the determination of adenine nucleotides. The initial values (depleted) were determined for the depleted mitochondria prior to incubation with adenine nucleotides. Experimental details are given in section 2.

Table 1
Effects of inhibitors on mitochondrial adenine nucleotide accumulation

	AdN accumulated (nmol/mg protein) 10 min	20 min
No inhibitor	5.94 ± 0.43 (5)	11.27 ± 1.32 (4)
Oligomycin (1 μg/ml)	6.56 ± 1.76 (3)	13.21 ± 1.90 (3)
Antimycin A (1 μg/ml)	6.00 (1)	–
Dinitrophenol (40 μM)	4.60 (1)	–
Mersalyl (50 μM)	2.50 ± 0.46 (4)	3.24 ± 1.31 (2)
NEM (60 μM)	2.42 ± 0.40 (4)	–
No inhibitor, no phosphate	2.51 (1)	4.42 ± 1.24 (2)

Mitochondria were depleted of adenine nucleotides by treatment with pyrophosphate. The depleted mitochondria (~1.2 mg/ml) were incubated with 1 mM ATP as in section 2. Mitochondrial inhibitors were added or P_i was eliminated from the incubation medium as indicated. The incubation time was either 10 or 20 min. The mitochondria were subsequently washed and extracted for the determination of total adenine nucleotide content (AdN). Values given are means ± SE

inhibitors on adenine nucleotide accumulation in depleted mitochondria. Dinitrophenol slightly inhibited the accumulation, while antimycin A had no significant effect; these results were also observed when adenine nucleotide accumulation was measured utilizing [^{14}C]ATP (not shown). Oligomycin slightly enhanced the accumulation. Addition of respiratory substrate (glutamate + malate or succinate) did not significantly affect the accumulation (not shown). The greatest inhibitory effect was seen with the sulfhydryl reagents, *N*-ethylmaleimide and mersalyl. In addition, accumulation was inhibited when organic phosphate was eliminated from the incubation medium. Also, it was found that the accumulation was temperature dependent; the rate of adenine nucleotide accumulation at 4°C was only 20% of that observed at 30°C.

Fig.2 shows the Mg^{2+} dependence of adenine

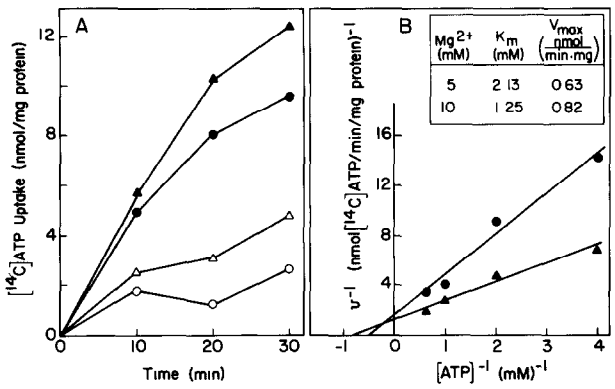

Fig.2. Mg^{2+} dependence of adenine nucleotide accumulation in depleted mitochondria. (A) Mitochondria (~1.2 mg/ml) were incubated with 1 mM [^{14}C]ATP in the presence of 50 μM atractyloside. The incubation conditions were as in section 2 under 'adenine nucleotide loading', except that the reloading was done in the absence (○) of $MgCl_2$ or in the presence of 3 mM (△), 5 mM (●) or 10 mM (▲) $MgCl_2$. At the times indicated, 1 ml suspension was rapidly filtered through Millipore AAWP filters (pore size, 0.8 μm) and immediately washed once with 10 ml ice-cold, 150 mM NaCl. Radioactivity on the filters was determined by liquid scintillation counting. (B) Double reciprocal plots of rates of adenine nucleotide accumulation versus ATP concentrations. Mitochondria (~1.2 mg/ml) were incubated with 0.25, 0.5, 1.0 and 1.5 mM [^{14}C]ATP in the presence of 50 μM atractyloside. The other incubation conditions were as in section 2 under 'adenine nucleotide loading', except the $MgCl_2$ was either 5 mM (●) or 10 mM (▲). At timed intervals 1 ml suspension was rapidly filtered and washed as in fig.2A. The rates of uptake were calculated as the slopes determined by linear regression of the amount of label accumulated at 1, 2, 4 and 6 min. (Accumulation was linear over this time.)

nucleotide accumulation for depleted mitochondria. The rate and extent of accumulation was impaired in the depleted mitochondria if Mg^{2+} was absent during the incubation (fig.2A). Increasing Mg^{2+} from 5–10 mM slightly decreased the app. K_m and slightly increased the V_{max} (fig.2B). Due to low rates of accumulation, kinetic data were not obtained for the depleted mitochondria in the absence of Mg^{2+}.

4. Discussion

This study shows that isolated liver mitochondria can accumulate adenine nucleotides when incubated with physiologic concentrations of ATP (or ADP). Similarly, the adenine nucleotide content of perinatal rat liver mitochondria was increased after in vitro incubation with ATP [1,3]. The only other study of this nature with mammalian mitochondria is that in [8] where net uptake of massive amounts (100–200 nmol/mg protein) of adenine nucleotides during calcium accumulation was demonstrated in isolated rat liver mitochondria. Although it was not the major point of the study, some adenine nucleotide accumulation was reported in the absence of added calcium [8]. In [9–11] the net uptake of ADP in corn mitochondria was reported.

The net uptake of adenine nucleotides was not mediated by the adenine nucleotide carrier. The inhibition by NEM and mersalyl, as well as the necessity for added phosphate, suggests that the phosphate–hydroxyl exchanger may play a significant role in the accumulation mechanism. Perhaps intramitochondrial phosphate exchanges with extramitochondrial ATP (or ADP). The slower rate of adenine nucleotide accumulation in the absence of added Mg^{2+} may indicate that ATP is transported across the inner membrane as a Mg–ATP complex. However, the Mg^{2+} effect may be due to enhanced membrane integrity; membrane-bound Mg^{2+} may have been lost during the pyrophosphate treatment.

Although not presented here, one of the significant aspects of our work is the observation that the accumulation of adenine nucleotides is associated with enhanced respiratory functions [6]. Adenine nucleotide accumulation or rat liver mitochondria during perinatal development has been shown to be accompanied by increased respiratory activity [1,3]. Moreover, glucagon injection of adult rats has been reported to increase the adenine nucleotide content of liver

mitochondria [4,5]. It had been shown that the respiratory rate of liver mitochondria was enhanced after glucagon administration [12]. Therefore, the experimental design presented here may provide an in vitro model for studying the mechanism and regulatory effects of adenine nucleotide accumulation in mitochondria.

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