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ADENINE NUCLEOTIDE EFFLUX IN MITOCHONDRIA INDUCED BY INORGANIC PYROPHOSPHATE

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The efflux of mitochondrial adenine nucleotide which is induced by addition of PP_i to suspensions of rat liver mitochondria has been investigated. This efflux of adenine nucleotide is greatly stimulated by the uncoupler FCCP at 1 μM, V_{max} being 6.7 nmol/min per mg protein as compared to 2.0 nmol/min per mg protein in its absence. The depletion process is inhibited by carboxyatractyloside. The K_m for PP_i of 1.25 mM is essentially unchanged when uncoupler is added. Quantitation of the individual adenine nucleotide species (ATP, ADP and AMP) and their relationship to the rate of efflux suggests that ADP is the predominant species being exchanged for PP_i.

Introduction

It has recently been demonstrated that mitochondria can be depleted almost entirely of their adenine nucleotides by a process that is carboxyatractyloside sensitive [1]. The adenine nucleotide translocase which catalyses the exchange of ADP and ATP across the inner mitochondrial membrane is strongly inhibited by carboxyatractyloside and its mechanism has been extensively studied (for reviews see Refs. 2, 3). Less is known however about the pyrophosphate (PP_i) induced efflux of nucleotides which apparently takes place via the same protein [1]. This process could represent a means of altering the intramitochondrial pool of adenine nucleotides by transferring nucleotides between the mitochondrial and cytosolic pools. An effect of exogenous ADP stimulated respiration on the intramitochondrial adenine nucleotide pool size has been reported [1].

The aim of this study was to characterize the PP_i induced efflux from isolated rat liver mitochondria and to identify the species being exchanged for inorganic PP_i. The data show that mitochondria can be depleted (approx. 80%) of their total adenine nucleotides by exchange for PP_i, in a process that is stimulated by FCCP. ADP appears to be the predominant species exchanged for PP_i.

Abbreviations: PP_i, pyrophosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Materials and Methods

1. Isolation of mitochondria. Mitochondria were isolated from the livers of male Sprague-Dawley rats (180–220 g) in sucrose/Tris/EGTA as described by Aprille and Asimakis [1], finally washed and resuspended in 250 mM sucrose/1 mM Tris-HCl (pH 7.4) to approx. 30 mg protein/ml.

2. Adenine nucleotide depletion. Mitochondria (30 mg/ml) were preincubated for 5 min at 4°C with 2 μCi [2-^{3}H]ATP (spec. act. 250 mCi/mmol), diluted with 4 vols. of sucrose/Tris/EGTA medium, washed once by centrifugation and finally suspended into sucrose/Tris-HCl (pH 7.4). Aliquots of protein were then diluted into 225 mM

sucrose/7.5 mM KCl/5 mM Tris-HCl (pH 7.4) containing 5 mM malate/glutamate at protein concentrations specified in the legend and placed in a 16°C water bath with constant shaking. Adenine nucleotide efflux was initiated by addition of sodium pyrophosphate. Samples (300 μl) were withdrawn at the indicated time intervals and placed in ice-cold centrifuge tubes containing carboxyatractyloside to give a final concentration of 20 μM. The samples were then centrifuged (Beckman Model B microfuge). The mitochondria passed through a layer (approx. 30 μl) of silicone oil (specific gravity 1.07, General Electric, Waterford, NY) which was at the bottom of the tube and thus were separated from the supernatant fraction. Radioactivity in the supernatant fractions and pellets was counted in a Searle Delta 300 Liquid scintillation counter after solubilization in aqueous Scintillant (Amersham).

3. Measurements of individual nucleotides. Mitochondria (4 mg/ml protein) suspended in sucrose/KCl/Tris buffer were equilibrated at 16°C in a shaking water bath. Adenine nucleotide efflux was initiated by addition of 4 mM PP_i and samples (500 μl) were withdrawn at indicated time intervals and placed in 1.2 ml centrifuge tubes. Each tube contained 100 μl of a 4% trichloroacetic acid/4% NaCl solution over which was layered 400 μl silicone oil (s. g. 1.07). These tubes were then centrifuged for 2 min in the microfuge. Adenine nucleotide in the trichloroacetic acid extracts were measured using high-pressure liquid chromatography (HPLC) over a Whatman PXS-1025 SAX ion-exchange column and an ultraviolet detector set to measure the absorbance at 260 nm. The elution was by a gradient of 0.01–0.4 M phosphate buffer, pH 4.5. The concentrations of the individual nucleotides were calculated by comparison with the elution pattern of known mixtures of adenine nucleotides.

Protein was measured with biuret reagent using bovine serum albumin as the standard.

Results

1. The rate of adenine nucleotide efflux from mitochondria treated with pyrophosphate

Mitochondria which had been loaded with radioactive adenine nucleotide were incubated at 16°C and the release of radioactivity from the mitochondria following addition of PP_i was measured (Fig. 1). Addition of 1 mM or 10 mM PP_i caused release of approx. 36% and 55%, respectively, of the total adenine nucleotides in 5 min. The presence of uncoupler (1 μM FCCP) did not by itself cause any significant efflux of adenine nucleotides though it greatly increased the rate of the PP_i-induced efflux. When carboxyatractyloside was included in the depletion medium no significant release of nucleotides was observed. Respiratory chain inhibitors, e.g., rotenone and cyanide did not alter the nucleotide efflux rate. Oligomycin, an inhibitor of the energy transfer pathway also had no significant effect on the measured adenine nucleotide efflux. Addition of NaF to inhibit pyrophosphatase activity did not alter the amount of adenine nucleotide released.

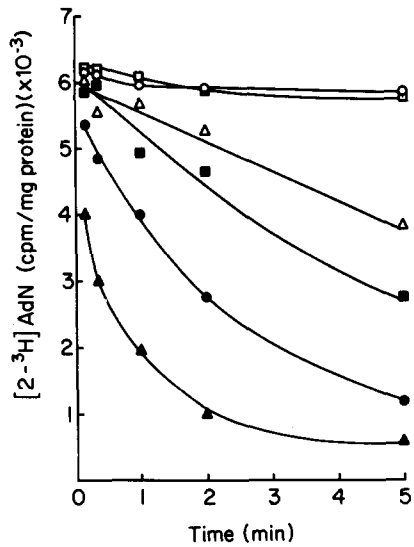

Fig. 1. Efflux of adenine nucleotides (AdN) in isolated rat liver mitochondria. Rat liver mitochondria (1.5 mg/ml) were treated as described in the Methods. Efflux measurements were started by the addition of PP_i and incubated at 16°C with constant shaking. Samples (300 μl) were withdrawn at the time intervals indicated into tubes containing 20 μM carboxyatractyloside at 4°C prior to centrifugation through a layer of silicone oil. The data are for one of three identical experiments performed. The results for the three experiments are within 5% of each other. Additions: ▲——▲, 10 mM PP_i + 1 μM FCCP; ●——●, 1 mM PP_i + 1 μM FCCP; ■——■, 10 mM PP_i; △——△, 1 mM PP_i; □——□, 20 μM carboxyatractyloside + 10 mM PP_i + 1 μM FCCP; ○——○, control.

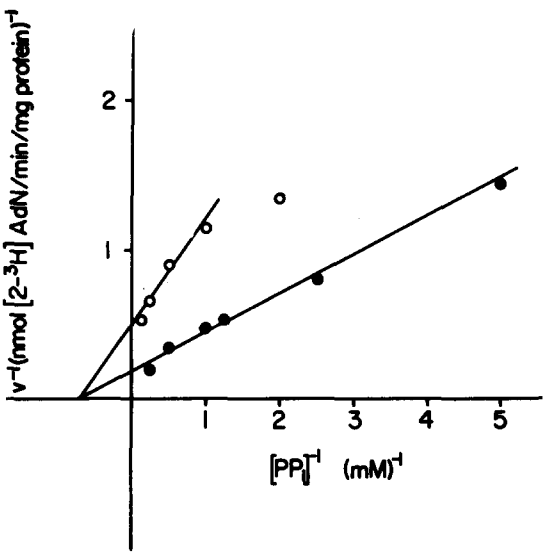

Fig. 2. Kinetic analysis of adenine nucleotide (AdN) efflux. Double-reciprocal plots were constructed from the initial rates of efflux with (●———●) or without (○———○) FCCP. Conditions for the measurement were the same as described in Fig. 1.

2. Kinetics of the adenine nucleotide release

Fig. 2 shows the apparent K_m and V_{max} values for adenine nucleotide depletion calculated from the initial linear rates of efflux. The maximal velocity for adenine nucleotide efflux was 2.0 nmol/min per mg protein and increased to 6.7 nmol/min per mg protein in the presence of FCCP in agreement with the faster depletion observed in Fig. 1. The apparent K_m (1.25 mM) remained the same under both sets of conditions. At low pyrophosphate concentrations in the absence of uncoupler, the measured efflux rates are higher than expected for a simple Michaelis system. The efflux rates in this region are low and a trace of PP_i-independent efflux could contribute to the observed data, but, this is unlikely to be the only explanation.

3. Intramitochondrial adenine nucleotide content during the time course of depletion by PP_i

The intramitochondrial adenine nucleotide content was measured both at 10 min and various time intervals after addition of 4 mM pyrophosphate as described in Materials and Methods. Upon centrifugation the mitochondria passed through the silicone oil layer and the enzymatic activities were quenched in trichloroacetic acid. The ATP, ADP and AMP contents of the trichloroacetic acid layer were then measured and these values, expressed as nmol/mg protein, are given in Tables I and II.

The total adenine nucleotide pool decreased from approx. 10.7 to 2.6 nmol/mg for untreated mitochondria with greater decreases in ADP (3.45 nmol/mg) and AMP (2.75) than in ATP (1.93). When 1 μM FCCP was present the total adenine nucleotide content decreased even further to 1.31 nmol/mg, but again the decrease in ADP (5.2 nmol/mg) was greater than that for AMP (1.91) or ATP (1.54). A similar pattern of adenine nucleotide depletion was observed when aliquots of PP_i-treated mitochondria in the presence of

TABLE I

INTRAMITOCHONDRIAL ADENINE NUCLEOTIDE CONTENT

Mitochondria (4 mg protein/ml) were treated as described in Materials and Methods. The concentrations of the individual adenine nucleotides in the mitochondria were determined by HPLC after 10 min incubations with PP_i.

Sample	ATP (nmol/mg)	ADP (nmol/mg)	AMP (nmol/mg)	Total adenine nucleotides (nmol/mg)
Mitochondria	3.18	4.46	3.07	10.72
Mitochondria + 1 μM FCCP	1.65	5.78	3.24	10.76
Mitochondria + 4 mM PP_i	1.25	1.01	0.32	2.58
Mitochondria + 1 μM FCCP + 4 mM PP_i	0.11	0.58	0.64	1.33

TABLE II

INTRAMITOCHONDRIAL ADENINE NUCLEOTIDE CONTENT DURING PYROPHOSPHATE DEPLETION

Mitochondria (4 mg/ml) were treated as described in Table I, except that samples were withdrawn at 20 s, 2 and 5 min time intervals after treatment with PP_i.

Sample	Time (s)	ATP (nmol/mg)	ADP (nmol/mg)	AMP (nmol/mg)	Total adenine nucleotides (nmol/mg)
Mitochondria + 1 μM FCCP	0	2.0	6.07	3.0	11.07
Mitochondria + 1 μM FCCP + 4 mM PP_i	20	1.83	0.86	2.02	4.71
Mitochondria + 1 μM FCCP + 4 mM PP_i	120	1.33	0.61	1.02	2.96
Mitochondria + 1 μM FCCP + 4 mM PP_i	300	0.67	0.29	0.63	1.59

FCCP (1 μM) were withdrawn at 20 s, 2 min and 5 min intervals. The data show that ADP appears to be the first species depleted, decreasing to 14.1% of the initial concentration after 20 s. AMP is next, decreasing to 67.3%, followed by ATP, which dropped to 91.5% of the total amount after 20 s.

Discussion

In this study we have characterized another aspect of the adenine nucleotide translocator. Our data have confirmed the report by Aprille and Asimakis [1] that PP_i is capable of exchanging for intramitochondrial adenine nucleotides by a carboxyatractyloside-sensitive mechanism. Investigation of the dependence of the rate of adenine nucleotide efflux on pyrophosphate concentration showed saturation behavior, with an apparent K_m for pyrophosphate of 1.25 mM. In the presence of uncoupler the K_m remained the same, however the V_{max} increased from 2 to 6.7 nmol/min per mg protein. The PP_i presumably substitutes for ATP^{4-} or ADP^{3-} in the translocase mechanism, the latter being most consistent with the ionization constant for PP_i. The presence of a transmembrane electrical potential, negative inside, is reported to be energetically coupled to the export of ATP^{4-} in exchange for ADP^{3-} [2]. Uncoupler, by dissipating this membrane potential, would be expected to inhibit net efflux of adenine nucleotides if it occurred through an exchange of ATP^{4-} for PP_i^{3-}. Adenine nucleotide analysis indicated that in the presence of FCCP the intramitochondrial ADP increased markedly while ATP decreased. Thus the FCCP induced increase in efflux suggests that PP_i exchanges primarily for ADP.

Depleted mitochondria retain approx. 1–2.5 nmol adenine nucleotide per mg protein, the ratio of ATP:ADP:AMP being 1.25:1.0:0.32. Analogous ratios of remaining nucleotides in the presence of FCCP are 0.1:0.6:0.6. The former values are slightly higher than those reported by Aprille and Asimakis [1], but their experiments were performed at 30°C, whilst 16°C was used in the present work so as to decrease the rate of efflux and permit more accurate kinetic measurements. In fact, no significant PP_i-induced adenine nucleotide efflux occurs in minutes at 0–4°C (data not shown). The remaining intramitochondrial nucleotides may represent species tightly bound to the ATPase as suggested by Stoner and Sirak [7]. It has been suggested that in vivo mitochondria are capable of synthesizing pyrophosphate in competition with adenine nucleotide phosphorylation [8], and that the reverse process could operate for uptake of nucleotides [7]. However, in normal cells the concentration of pyrophosphate has been reported to be approx. 12 μM [9], suggesting that for this mechanism to be operative in vivo the K_m values must be very different from that measured for adenine nucleotide efflux. Asimakis and Aprille [10] and Pollak and Sutton [11] observed adenine nucleotide uptake in depleted mitochondria to values above those for control mitochondria. We were, however, unable to observe large adenine nucleotide uptake except that reported to occur in

the presence of Ca^{2+} [12]. The reason for this discrepancy is under investigation.

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