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THE EXTENT OF MITOCHONDRIAL F_1-ATPase AND ADENINE NUCLEOTIDE CARRIER ACTIVITY WITH ϵ-ATP

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Summary

1. The use of 1,N^6-ethenoadenosine 5'-triphosphate (ϵ-ATP), a synthetic, fluorescent analog of ATP, by whole rat liver mitochondria and by submitochondrial particles produced via sonication has been studied.

2. Direct [^{3}H]adenine nucleotide uptake studies with isolated mitochondria, indicate the ϵ-[^{3}H]ATP is not transported through the inner membrane by the adenine nucleotide carrier and is therefore not utilized by the 2,4-dinitrophenol-sensitive F_1-ATPase (EC 3.6.1.3) that functions in oxidative phosphorylation. However, ϵ-ATP is hydrolyzed by a Mg^{2+}-dependent, 2,4-dinitrophenol-insensitive ATPase that is characteristic of damaged mitochondria.

3. ϵ-ATP can be utilized quite well by the exposed F_1-ATPase of sonic submitochondrial particles. This ϵ-ATP hydrolysis activity is inhibited by oligomycin and stimulated by 2,4-dinitrophenol. The particle F_1-ATPase displays similar K_m values for both ATP and ϵ-ATP; however, the V with ATP is approximately six times greater than with ϵ-ATP.

4. Since ϵ-ATP is a capable substrate for the submitochondrial particle F_1-ATPase, it is proposed that the fluorescent properties of this ATP analog might be employed to study the submitochondrial particle F_1-ATPase complex, and its response to various modifiers of oxidative phosphorylation.

Introduction

ϵ-ATP (1,N^6-ethenoadenosine 5'-triphosphate), a synthetic fluorescent analog of ATP has been shown to be a capable substrate for several soluble enzymes [1]. These include hexokinase (EC 2.7.1.1), 6-phosphofructokinase (EC 2.7.

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Abbreviations: ϵ-ATP, 1, N^6-ethenoadenosine 5'-triphosphate; AdN, adenine nucleotide.

1.11) and adenylate kinase (EC 2.7.4.3). It is also a good substrate for the ATPase (EC 3.6.1.3) of rat liver plasma membranes [2], but is not significantly hydrolyzed by the chloroplast affiliated ATPase [3]. Recently, Bârzu et al. [4] have shown that ϵ-ATP is hydrolyzed by both osmotically shocked mitochondria and by lubrol-particles.

Mitochondria contain an AdN translocase which transports external ATP or ADP through the inner membrane in an equimolar exchange with internal AdN [5—8]. While the translocase is capable of slowly exchanging analogs of ATP and ADP which contain alterations in the phosphate chain [9,10] or in the ribose [11], it maintains a strong requirement for an intact adenine base and at least two phosphate groups [12]. In order for an AdN to be utilized by the F_1-ATPase that is involved in oxidative phosphorylation, it must first be transported through the inner membrane by the highly specific AdN translocase to the vicinity of the enzyme. In contrast, since most submitochondrial particles produced by sonication possess an exposed F_1-ATPase [13,14], AdN hydrolysis can occur without prior transport. The sonic particle F_1-ATPase is not very substrate specific and can utilize GTP, ITP and dATP rather well [15,16].

The results presented here indicate that in whole mitochondria, ϵ-ATP is not a substrate for the AdN carrier and therefore cannot be utilized by the compartmentalized F_1-ATPase. However, the analog is readily hydrolyzed by the membrane-bound submitochondrial particle F_1-ATPase. A portion of these results has been presented elsewhere [17].

Methods

Liver mitochondria were isolated from male Long-Evans rats according to a procedure modified from Kielley and Kielley [18] with 0.25 M sucrose/1 mM EDTA employed as the isolation medium. The tightness of coupling of each mitochondrial preparation was routinely assayed immediately prior to, and following the experimental incubations of a given day using the graphic method described by Estabrook [19]. These incubations were performed with a Clark-type oxygen electrode at 28°C with 7.5 mM succinate as substrate under conditions similar to those described by Coleman [20] except for the presence of 100 mM sucrose in the incubation mixture. An average ADP/0 ratio of 1.63 and respiratory control ratio of 4.07 were obtained. Protein and orthophosphate were determined as described previously [21].

Submitochondrial particles were prepared from freshly isolated rat liver mitochondria. The mitochondrial pellet was resuspended in 1 ml of cold deionized H_2O per g of original tissue. Our sonication procedure was similar to that employed by Weidemann et al. [22]. The suspension was subjected to 5-s sonication bursts (Branson Sonifier, Model W185, max. power output) which were interspersed with 55 s of cooling in an ice/acetone bath. The total sonication time was 1 min during which the sample temperature was maintained at 0—5°C. The sonicated sample was centrifuged at 25 000 $\times g$ for 10 min at 0°C and the resulting supernatant was then centrifuged at 108 000 $\times g$ for 25 min at 0°C. The sonic particle pellet was resuspended in cold deionized H_2O containing 15% (v/v) dimethylsulfoxide to a protein concentration of approx. 15—25 mg/ml, and stored at —80°C.

The AdN hydrolysis incubations with whole mitochondria have been previously described [21]. All AdN hydrolysis incubations, employing either mitochondrial or sonic particle preparations, were terminated with an equal volume of 6% (v/v) $HClO_4$ and treated as described previously [21]. The reaction mixtures used for the determination of the K_m and V of the sonic particle ATPase for ATP and ϵ-ATP consisted of 10 mM Tris/maleate buffer (pH 7.2), 1.0 mM $MgCl_2$, and a range of AdN concentrations. In addition, the ATP incubations contained 0.20 mg sonic particle protein/ml, whereas the ϵ-ATP incubations contained 0.51 mg sonic particle protein/ml. Controls were run for endogenous sonic particle orthophosphate and non-enzymatic hydrolysis of each AdN concentration. Incubations were initiated by the addition of AdN and run for 10 s at 28°C. Under the conditions employed, the observed reaction velocities were linear for at least 20 s with each AdN concentration. Thus, the reaction rate after 10 s was taken as an initial velocity.

The uptake of ^3H-labeled AdN into mitochondria was measured by a method similar to that described by Winkler et al. [23]. Incubations were initiated by the addition of ^3H-labeled AdN to the reaction mixtures described in Table I. Reactions were terminated by rapidly filtering 0.85-ml aliquots of the incubation mixture through nitrocellulose filters (S and S; pore diameter 0.45 μm) which were fastened in a vacuum manifold. The mitochondria on the filter were then washed three times with 1-ml aliquots of a solution containing 100 mM sucrose and 20 mM Tris/maleate buffer (pH 7.2). The filters were dried and counted in a liquid scintillation cocktail that consisted of 0.13% (w/v) 2,5-diphenyloxazole in toluene.

The synthesis of ϵ-ATP was performed essentially by the method of Secrist et al. [1] employing the modifications that were previously described [21]. ϵ-[2-^3H]ATP was synthesized from [2-^3H]ATP and chloroacetaldehyde. Under these conditions it was necessary to allow the reaction to continue 2—3 days in order to insure completion. The purity of ϵ-ATP with respect to unmodified ATP was approx. 97% and was determined spectrometrically as described previously [21].

Results and Discussion

The substrate capability of ϵ-ATP for the AdN carrier and the F_1-ATPase in isolated mitochondria

The extent of the transport of [2,8-$^3H_2$]ATP and ϵ-[2-^3H]ATP into mitochondria was measured as indicated in Table I. The low level of the observed ϵ-[2-^3H]ATP counts, together with the inability of atractyloside to significantly decrease this minimal radioactivity, indicates that ϵ-ATP is not transported by the AdN carrier into mitochondria.

Initially, the purity of ϵ-[^3H]ATP presented serious problems, for in the presence of small but significant amounts of unmodified [^3H]adenine nucleotides which remained as impurities in the ϵ-[^3H]ATP product, label was transported into mitochondria by an atractyloside-sensitive process [17]. The application of more stringent synthetic conditions resulted in a more highly purified ϵ-product with which transport was not observed. These results re-emphasize

TABLE I

TRANSPORT OF ^{3}H-LABELED AdN INTO WHOLE MITOCHONDRIA

The reaction mixtures contained 20 mM Tris/maleate buffer (pH 7.2), 100 mM sucrose, 5 mM $MgCl_2$, 0.6 mM ^{3}H-labeled AdN, an average of 1.36 mg mitochondrial protein/ml, and 3.3 mM atractyloside where indicated. Temperature was 28°C. Reactions were initiated by the addition of ^{3}H-labeled AdN and terminated as described in Methods. During atractyloside incubations, mitochondria were preincubated with the inhibitor for 2 min before reactions were triggered by the addition of ^{3}H-labeled AdN. The specific activities of [2,8-$^3H_2$]ATP and ϵ-[2-^{3}H]ATP were $1.37 \cdot 10^{11}$ and $1.41 \cdot 10^{11}$ cpm/mol, respectively. Data were normalized to the same specific activity for the two [^{3}H]adenine nucleotides. Each value represents the mean of five repetitions.

Substrate (0.6 mM)	Incubation time (min)	Uptake (cpm/mg)	
		Minus atractyloside	Plus atractyloside
[^{3}H]ATP	1	630	78
ϵ-[^{3}H]ATP	1	51	36
[^{3}H]ATP	18	684	347
ϵ-[^{3}H]ATP	18	95	106

the high affinity of the AdN translocase for unmodified ATP and ADP and are in agreement with the work of others [4,24—26].

We have previously reported that in the presence of 5 mM $MgCl_2$, appreciable ϵ-ATP hydrolysis occurred which was inhibited by atractyloside, but was insensitive to 2,4-dinitrophenol [21]. Subsequent experiments confirmed these results and showed that ϵ-ATP hydrolysis maintains a strong requirement for exogenous Mg^{2+}, whereas ATP hydrolysis does not. Employing 10 mM AdN, the ϵ-ATP hydrolysis activity dropped from 18 nmol $P_i \cdot min^{-1} \cdot mg^{-1}$ in the presence of Mg^{2+} to 2 nmol $P_i \cdot min^{-1} \cdot mg^{-1}$ in its absence, whereas the ATP hydrolysis activity was 57 nmol $P_i \cdot min^{-1} \cdot mg^{-1}$ under both conditions. It is well documented [27—30] that structurally damaged mitochondria display a Mg^{2+}-sensitive, 2,4-dinitrophenol-insensitive ATPase activity. Since most preparations of freshly isolated mitochondria probably contain at least a small percentage of disrupted organelles, it would appear that these damaged mitochondria may be responsible for the observed ϵ-ATP hydrolysis. Bârzu et al. [4] have reached similar conclusions. In light of the fact that ϵ-ATP is not transported into mitochondria one can only speculate as to the mechanism of the atractyloside inhibition of analog hydrolysis.

The substrate capability of ϵ-ATP for the exposed F_1-ATPase in sonic submitochondrial particles

Submitochondrial particles produced by sonication consist of an everted inner mitochondrial membrane in which most of the F_1-ATPase molecules face the external environment [13,14]. Thus adenine nucleotides added to a suspension of these particles are immediately placed into the microenvironment surrounding the F_1-ATPase. Table II indicates that in the presence of 10 mM AdN and 5 mM $MgCl_2$, ϵ-ATP is an excellent substrate for the sonic particle F_1-ATPase. The hydrolysis of both ATP and ϵ-ATP was inhibited appreciably by oligomycin. Fig. 1 demonstrates that various concentrations of 2,4-dinitrophenol

TABLE II

OLIGOMYCIN INHIBITION OF AdN HYDROLYSIS BY SONIC PARTICLE ATPase IN THE PRESENCE OF 5 mM $MgCl_2$

Reactions were initiated by the addition of sonic particles, and terminated after 18 min as described in Methods. The incubation mixtures contained 10 mM Tris/maleate buffer (pH 7.2), 5.0 mM $MgCl_2$, 10 mM AdN, and an average of 1.45 mg sonic particle protein/ml. Temperature was 28°C. Absolute dimethylsulfoxide was the solvent for oligomycin and represented 1.5% of the total incubation volume. Controls contained 1.5% (v/v) dimethylsulfoxide without oligomycin. Also, controls were run for orthophosphate endogenous to the sonic particle preparation as well as that arising from non-enzymatic hydrolysis of AdN. The values in parentheses represent the number of repetitions for a given incubation.

Substrate (10 mM)	Oligomycin (μg/ml)	P_i released (nmol $P_i \cdot min^{-1} \cdot mg^{-1}$)	Inhibition (%)
ATP (control)	—	403 (5)	—
ATP	1.5	118 (5)	71
ϵ-ATP (control)	—	261 (5)	—
ϵ-ATP	1.5	69 (5)	74

cause considerable enhancement of both ATP and ϵ-ATP hydrolysis. This effect is consistent with its ability to uncouple oxidative phosphorylation and with its role as an activating oxy anion [31].

Lineweaver-Burk analysis of initial rate studies performed with various concentrations of AdN indicated that the K_m values for the two substrates are similar (for ATP: 0.17 mM; for ϵ-ATP: 0.12 mM). However, the maximal velocity

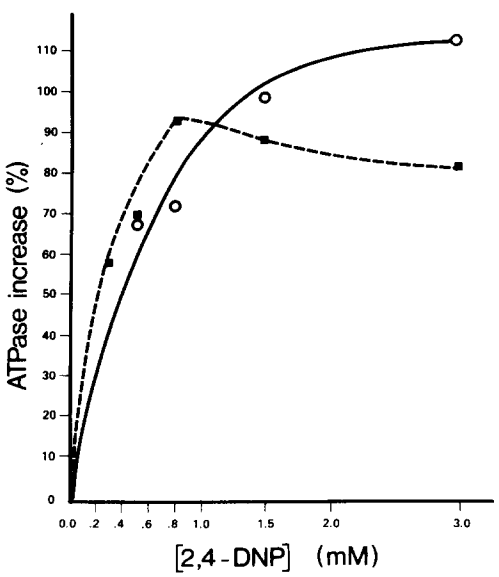

Fig. 1. Effect of 2,4-dinitrophenol on AdN hydrolysis in submitochondrial particles. Conditions as described in Table II with the following modifications. Dimethylsulfoxide was omitted from all incubations. The incubation mixtures contained 10 mM Tris/maleate buffer (pH 7.2), 0.1 mM $MgCl_2$, 10 mM AdN, an average of 1.47 mg sonic particle protein/ml, and various concentrations of 2,4-dinitrophenol (2,4-DNP) as indicated. Incubations were repeated an average of five times. o——o, ATP; ■——■, ϵ-ATP.

observed with ATP was approximately six times the value observed with ϵ-ATP (with ATP: 3.56 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}; with ϵ-ATP: 0.62 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}). Thus, although both adenine nucleotides are capable substrates for the F_1, the kinetic characteristics of the two hydrolysis reactions differ appreciably.

We conclude that ϵ-ATP is a good substrate for the sonic particle ATPase and may serve as a useful fluorescent probe to study this membrane-bound F_1-ATPase complex and its response to various modifiers of oxidative phosphorylation.

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