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ADENINE NUCLEOTIDE TRANSLOCATION IN PLANT MITOCHONDRIA

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Key Word Index—*Zea mays*; Gramineae; corn; mitochondria; adenine nucleotide carrier; adenosine diphosphate; atractyloside.

Abstract—The rapid translocation of external ADP-[^{14}C] by corn mitochondria is inhibited by high concentrations of atractyloside with enhanced inhibition occurring in the presence of Mg^{2+}. This translocation is also inhibited by AMP or ATP but CDP, GDP, IDP or UDP have little effect. Backward exchange of internal ADP-[^{14}C] occurs in the presence of AMP, ADP or ATP but is not promoted by other nucleoside diphosphates. It is suggested that the adenine nucleotide (AdN) carrier is specific for ADP and ATP and that apparent translocation of AMP is a result of adenylate kinase activity. The translocated ADP can be separated into 3 components: (1) atractyloside-insensitive binding; (2) carrier-bound ADP saturated at *ca* 30 μM external ADP; and (3) exchanged ADP saturated as *ca* 5 μM external ADP. It is suggested that the adenine nucleotide carrier of plant mitochondria possesses similar properties to the classical carrier of vertebrate mitochondria.

INTRODUCTION

The mitochondrial F_1 ATPase, responsible for ATP synthesis in oxidative phosphorylation, is located on the inner surface of the inner mitochondrial membrane [1]. The required substrates are translocated across the inner membrane by means of the Pi^-/OH^- [2] and ADP^{3-}/ATP^{4-} [3] antiporters. The mechanism of action of the adenine nucleotide (AdN) carrier of vertebrate mitochondria is the most completely understood of the various mitochondrial carriers due largely to use of the specific inhibitor atractyloside, a glycoside isolated from the thistle, *Atractylis gummifera* L. [4]. Atractyloside competitively inhibits the binding of ADP and ATP to the AdN carrier [3]. However, oxidative phosphorylation in plant mitochondria is only sensitive to atractyloside at relatively high concentrations [5–7]. Moreover, uptake of ADP-[^{14}C] in jerusalem artichoke mitochondria is slow and resistant to atractyloside [8]. This was ascribed to a low endogenous AdN pool together with non-specific nucleotide binding to the membranes. Subsequent work has though indirectly demonstrated the existence of an AdN carrier in artichoke mitochondria [6].

Previous work from this laboratory [7] suggested that the ADP-induced contraction of non-energised corn mitochondria reflected a change in orientation of the AdN carrier in the inner mitochondrial membrane. This paper directly demonstrates both the binding and exchange of ADP-[^{14}C] in these mitochondria and shows some common properties between the AdN carriers of plant and vertebrate mitochondria.

RESULTS AND DISCUSSION

The translocation of ADP-[^{14}C] in corn mitochondria is essentially complete in < 15 sec (Fig. 1). The rate of ADP translocation is faster than for artichoke mitochondria [8] and is comparable to rates obtained with vertebrate mitochondria when sampled by Millipore filtration [9]. Translocation of ADP is substantially inhibited by atractyloside present at the start of the experiment and termination of translocation by the addition of atractyloside removes a component of the translocated ADP which is assumed to represent ADP bound to the AdN carrier [10].

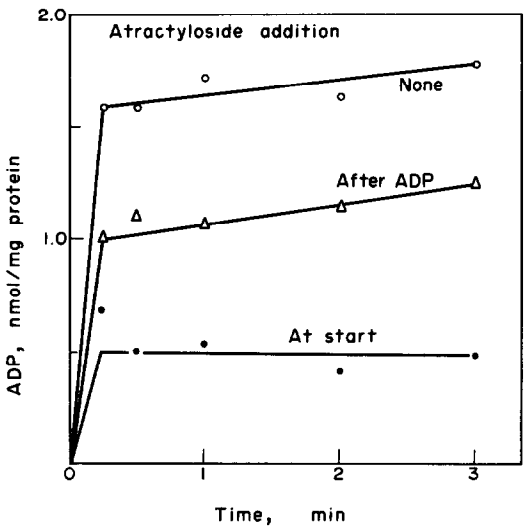

Fig. 1. The time course of ADP translocation. Reaction medium and conditions as in the Experimental except for the addition of 5 mM $MgSO_4$. Uptake of ADP (100 μM external concentration) was terminated either by the addition of atractyloside (770 nmol/mg protein) and immediate filtration (△) or by the initiation of filtration in samples untreated (○) or pre-treated with atractyloside (●).

Inhibition of ADP translocation by atractyloside in corn mitochondria is enhanced by the presence of Mg^{2+} (Fig. 2) as in vertebrate mitochondria and maximal

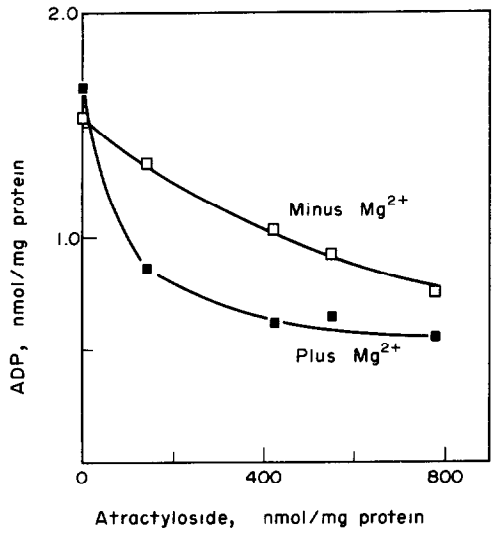

Fig. 2. The translocation of ADP as a function of atractyloside concentration in the presence and absence of Mg^{2+}. Reaction medium and conditions as in Fig. 1.

inhibition occurs at *ca* 400 nmol atratyloside/mg protein compared with *ca* 1 nmol atractyloside/mg protein in vertebrate mitochondria [9]. Previous work with corn mitochondria has shown maximal inhibition of both oxidative phosphorylation and ADP-induced contraction occurring at *ca* 50 nmol atractyloside/mg protein [7]. These different sensitivities to atractyloside in corn mitochondria presumably reflect mitochondrial protein concentration differences between the previous work [7] using 0.10–0.13 mg protein/ml and the present ADP-[^{14}C] translocation experiments at 1.30–1.80 mg protein/ml. There is indirect evidence for the enzymatic destruction of atractyloside in vertebrate mitochondria [10] and the relative insensitivity of the plant mitochondrial AdN carrier to atractyloside has been suggested to be due to a glucosidase associated with the mitochondria [6] which would release the aglycone, atractyligenin, a less potent inhibitor than atractyloside [11]. An increased mitochondrial protein concentration would, therefore, result in enhanced enzymatic destruction of atractyloside. Alternatively, plant mitochondria could possess atractyloside binding sites, other than the AdN carrier, which are greatly in excess to similar sites in vertebrate mitochondria [12].

Table 1. Specificity of the AdN carrier as measured by inhibition of external ADP-[^{14}C] translocation and promotion of backward exchange of internal ADP-[^{14}C]

Nucleotide	*ADP translocation (% inhibition)	†Backward Exchange (%)
AMP	44	53
ADP	—	54
ATP	86	68
CDP	− 2	− 2
GDP	3	10
IDP	11	4
UDP	10	13

*Reaction medium as in the Experimental with the addition of 1 mM EDTA (pH 6.5). Control value of ADP translocation (50 μM external concentration) was 1.08 nmol ADP/mg protein. Unlabelled nucleotides were present at 500 μM.
† Reaction medium and procedure as in the Experimental. Mitochondria contained 0.74 nmol ADP-[^{14}C]/mg protein.

Experiments have been carried out to determine the specificity of the AdN carrier in corn mitochondria and these are shown in Table 1. Firstly, the translocation of external ADP-[^{14}C] is substantially inhibited by a 10-fold excess of ATP. Less effective inhibition occurs with AMP, but CDP, GDP, IDP or UDP have little or no effect. Secondly, the backward exchange of internal ADP-[^{14}C] is promoted by external AMP, ADP or ATP with little or no exchange caused by CDP, GDP, IDP or UDP.

The inactivity of nucleoside diphosphates other than ADP suggests an AdN-specific carrier. However, the effectiveness of AMP in apparently promoting exchange is puzzling as the vertebrate mitochondrial AdN carrier is specific for ADP and ATP only [3, 9]. The experiments described in Table 1 employed 1 mM EDTA which has been found to remove apparent AMP exchange in vertebrate mitochondria by inhibiting adenylate kinase activity [9]. The AdN-induced contraction of corn mitochondria, thought to represent a change in orientation of the AdN carrier in the inner membrane, can be driven by AMP in the absence of EDTA but is completely inhibited by 1 mM EDTA at AMP concentrations < 10 μM [7]. At higher AMP concentrations, some contraction does occur in the presence of EDTA presumably due to incomplete inhibition of adenylate kinase activity, known to occur in isolated corn mitochondria [13]. It is suggested that apparent AMP exchange (Table 1) is due to conversion of AMP to ADP or ATP by adenylate kinase located in the intermembrane space [14]. Increasing the EDTA concentration resulted in leakage of internal ADP-[^{14}C] in the absence of external nucleotide indicative of membrane damage (data not shown).

Work with vertebrate mitochondria has successfully differentiated between (1) exchanged AdN, located in the matrix which is resistant to actractyloside added *after* ADP or ATP and (2) carrier-bound AdN which is removed by the atractyloside addition [10]. Thus, measurement of AdN translocation in the absence (S_1) and presence of atractyloside (S_2) together with AdN translocation terminated by an atractyloside addition (S_3) gives:

$$\text{Exchanged AdN} = S_3 - S_2$$
$$\text{Carrier-bound AdN} = S_1 - S_3$$

The successful use of this approach with plant mitochondria depends on the competitive removal of AdN from the carrier sites by atractyloside. The translocation of ADP in cauliflower mitochondria is competitively inhibited by atractyloside [15] and, in corn mitochondria, atractyloside-inhibited State 3 respiration can be relieved by increasing the ADP concentration [5]. The effect of external ADP concentration on ADP translocation by corn mitochondria in the absence (S_1) or presence (S_2) of atractyloside with an additional treatment in which atractyloside is added *after* ADP (S_3) is shown in Fig. 3. The response to increasing ADP concentrations is similar to the situation in vertebrate mitochondria collected by centrifugation except that atractyloside-insensitive ADP binding in vertebrate mitochondria shows an approximate linear increase [10]. Atractyloside-insensitive ADP binding is thought to be primarily due to occupation of the sucrose accessible space by ADP-[^{14}C] [10] and, therefore, the low and approximately constant level of ADP binding in corn mito-

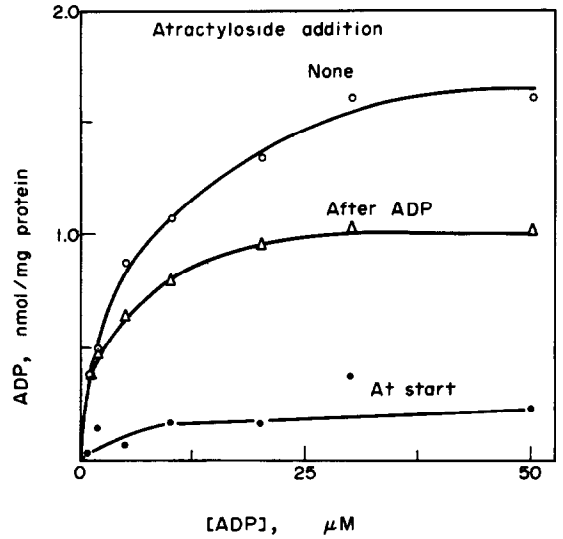

Fig. 3. The translocation of ADP as a function of ADP concentration. Reaction medium and conditions as in Fig. 1. At each ADP concentration, atractyloside (550 nmol/mg protein) was omitted (○), present from the start (●) or added 60 sec after initiation of uptake (△). All reactions were terminated 60 sec after initiation of uptake.

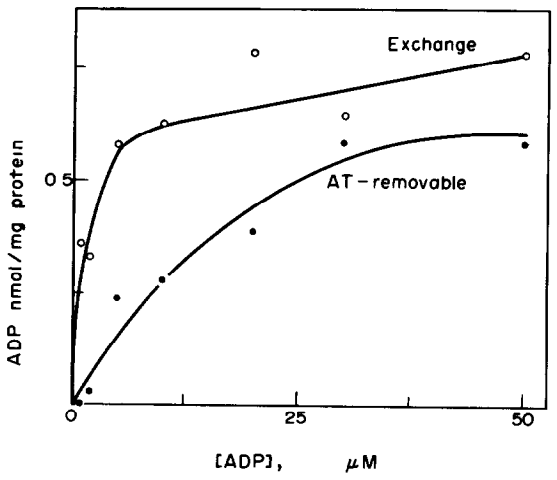

Fig. 4. The exchange and atractyloside-removable binding of ADP as a function of ADP concentration. The values are derived from the data in Fig. 3. Exchange = atractyloside termination minus atractyloside present at the start. Atractyloside-removable binding = total ADP translocation minus atractyloside termination.

chondria (Fig. 3) can be accounted for by the use of Millipore filtration which removes ADP-[^{14}C] from the sucrose accessible space [9].

The same data are used in Fig. 4 to distinguish between exchanged ADP and carrier-bound (atractyloside-removable) ADP. Exchange is saturated at *ca* 5 μM ADP in agreement with work on vertebrate mitochondria suggesting that exchange should be essentially independent of external AdN concentration [10]. Binding at the carrier sites is saturated at *ca* 30 μM ADP as in beef heart mitochondria which have been depleted of endogenous ADP + ATP [10].

Freshly-isolated vertebrate mitochondria are unsuitable for determining the level of carrier-bound ADP due primarily to a high internal level of ADP + ATP resulting in a high exchange of external ADP relative to the carrier-bound component [10]. However, a variety of procedures have been applied to reduce the internal ADP + ATP level thus increasing the proportion of carrier-bound ADP relative to exchanged ADP. For example, treatment with arsenate results in partial conversion of ADP + ATP to AMP and a lower ADP exchange (Table 2). The ease with which carrier-bound ADP can be determined in freshly-isolated corn mitochondria (Fig. 4) can be explained from the comparison of corn and vertebrate mitochondria presented in Table 2. Firstly, corn mitochondria possess a modest level of 2.5 nmol internal ADP + ATP/mg protein, a level similar to a previous independent determination for corn mitochondria [17]. Secondly, only a low proportion of the internal ADP + ATP of corn mitochondria participates in exchange for external ADP. The proportion of internal ADP + ATP participating in exchange has been found to vary unpredictably in vertebrate mitochondria [10].

The carrier-bound component of corn mitochondria is 0.55 nmol ADP/mg protein which approximates to comparable determinations for vertebrate mitochondria (Table 2). The difference in carrier-bound ADP between beef heart and rat liver mitochondria can be accounted for by different matrix protein contents; when expressed on the basis of cytochrome a content the determinations are more comparable [10].

The present results show significant similarities between the AdN carriers of plant and vertebrate mito-

Table 2. Comparison of internal adenine nucleotide content with exchange and binding of external ADP in corn and vertebrate mitochondria

Mitochondria	Treatment	Internal AdN content		External ADP translocation		
		AMP	ADP + ATP	Exchange	Exchange	Atractyloside-removable binding
		(nmol/mg protein)		(nmol/mg protein)	(%)	(nmol/mg protein)
Corn	Freshly-isolated	2.1	2.5	0.7	28	0.55
Beef heart	Freshly-isolated	1.4	2.6	2.5	98	0.45
Rat liver	Freshly-isolated	5.7	10.9	8.4	77	—
	Arsenate-depleted	9.0	1.8	0.7	41	0.13

The data for vertebrate mitochondria are taken from refs [10, 16].

chondria and emphasise the potential value of utilising plant mitochondria, with low internal ADP + ATP levels, for AdN carrier studies [18].

EXPERIMENTAL

Mitochondria were isolated from 6-day-old etiolated shoots of corn (*Zea mays* L. var. Kelvedon Glory) using a modified established method [19, 20]. Mitochondrial protein was determined using the Folin reagent [21] with bovine serum albumin (BSA) as a standard. Determination of mitochondrial ATP content was carried out using the luciferan–luciferase method in conjunction with a Packard liquid scintillation counter [22] as described by Bewley and Gwozdz [23]. Extracts of mitochondrial AdN were prepared as described by Jung and Hanson [17] and ADP and AMP were determined by conversion to ATP using pyruvate kinase and adenylate kinase [24]. Uptake of ADP-[^{14}C] was carried out using the Millipore filtration technique of Winkler *et al.* [9]. Mitochondrial suspension (50 μl containing 0.26–0.36 mg protein) in 400 mM sucrose was added to 150 μl of 200 mM sucrose, 20 mM imidazole–HCl (pH 6.5), 1 mg/ml BSA and ADP-[U-^{14}C] (*ca.* 3500 cpm ADP-[U-^{14}C]/nmol ADP) maintained at 0°. Reactions were terminated either by Millipore filtration (pore size, 0.45 μm) or by an atractyloside addition followed by immediate filtration. Samples (100 μl) were added to a filter previously pre-cooled by filtration of 5.0 ml of ice-cold 250 mM sucrose, 15 mM imidazole–HCl (pH 6.5) and washed with 10.0 ml of the same medium. The filters were added to liquid scintillation vials [25] and counted in a Packard liquid scintillation counter. Unless otherwise stated, ADP-[^{14}C] values are based on a 30 or 60 sec uptake period. Measurement of backward exchange was essentially according to Winkler *et al.* [9]. Mitochondrial protein (5.3 mg) was incubated in 1.0 ml of 200 mM sucrose, 20 mM imidazole–HCl (pH 6.5), 1 mM EDTA, 200 μM ADP-[U-^{14}C] at 0° for 20 min. The reaction was then made up to 40 ml with the same medium but minus ADP-[^{14}C] and centrifuged at 20000 *g* × 10 min at 0°. The supernatant was discarded, the inside of the tube wiped dry, and the pellet made up in 2.0 ml of 400 mM sucrose. Backward exchange was carried out in 5.0 ml of 200 mM sucrose, 20 mM imidazole–HCl (pH 6.5), 1 mg/ml BSA, 1 mM EDTA, 100 μM external nucleotide and mitochondrial protein (38 μg/ml) at 0° and was filtered at 5 min as described above.

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