Translocation of Adenine Nucleotides in the Mitochondria of Male Sterile and Male Fertile Sorghum*

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The translocation of ATP from the inside to the outside of the mitochondrial membrane has been studied in a male sterile (2219 A) and a male fertile (2219 B) line of sorghum. The translocation of ATP was found to be substantially lower in case of mitochondria from the male sterile line. The affinity of adenine nucleotides to the translocator proteins of the mitochondrial membrane was found to be almost one half in case of 2219 A as compared to 2219 B when the K_m for ATP-ADP was determined by two different assays. It is proposed that the inadequate supply of ATP in the cytosol resulting from its inefficient translocation may contribute to the male sterility in this line.

Introduction

In a respiring eukaryotic cell, the major part of ATP to be utilized in various energy consuming processes of the cell is supplied by mitochondria. The ATP produced in mitochondria is transported to the cytosol through the ADP-ATP translocator which catalyzes the translocation of one ADP molecule against one ATP molecule across the inner mitochondrial membrane in an energy-independent fashion [1, 2]. All mitochondria tested so far have an ADP-ATP translocation system. The translocator is a nuclear coded protein, and in beef heart mitochondria its molecular weight has been estimated to be approximately 30 kDa. The translocator exists as a dimer in the membrane. In the majority of the aerobic eukaryotic cells the ADP-ATP exchange is believed to be the most active transport system [2] and any deficiencies in this system might result in serious physiological consequences.

In an earlier communication we have reported that the respiratory activity of male sterile sorghum (line 2219 A) is lower as compared to its maintainer fertile line (2219 B) under field conditions [3]. We had postulated, therefore, that in male gamete forming cells where the requirement of ATP is comparatively much higher [4], lower respiratory rates might limit the supply of ATP resulting in male sterility. Secondly, in the inflorescence the chloroplast can not provide ATP directly as this tissue is usually devoid of functional chloroplasts. We have also reported that the mitochondrial electron transport is substantially lower in case of male sterile 2219 A as compared to the maintainer fertile 2219 B [5]. Also the phosphorylation of ADP to ATP driven by mitochondrial electron transport is lower in 2219 A as compared to 2219 B when the P : O ratio was determined at higher temperature (35 °C) [5]. Thus it appeared that the membrane-bound complexes carrying out energy transduction processes in mitochondria are less efficient in the male sterile line. The question of whether ATP synthesis alone is less efficient, or whether its translocation across the membrane through the ATP/ADP translocator is also affected was investigated in this study. The results demonstrated that the ATP translocation across the mitochondrial membrane is less efficient in the male sterile line as compared to the fertile line and that the affinity of the translocator for ATP/ADP in the male sterile line is almost one half that in the male fertile line.

Materials and Methods

Seeds of *Sorghum bicolor* Moench. L lines 2219 A (male sterile) and 2219 B (maintainer male fertile) were obtained from Sorghum Improvement Project, Hyderabad, India.

Mitochondria were isolated from 20–30 g of 5 day old etiolated coleoptiles using the basic procedure as described previously [6].

The mitochondrial pellet was suspended in 2 ml of the resuspending medium (mannitol, 0.4 M;

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HEPES, pH 7.2, 20 mM and BSA, 0.4%). For the experiment using radiolabelled ATP, the pellet was resuspended in 2 ml of MT buffer (mannitol, 0.4 M; triethanolamine, pH 7.2, 20 mM).

Intactness of the mitochondria was assayed using the succinate: cytochrome *c* oxidoreductase activity as described earlier [7]. For the intactness assay, 15 µl of the mitochondrial sample equivalent to 40–50 µg protein was added to 0.45 ml of assay buffer containing mannitol, 0.45 M; $MgCl_2$, 5 mM; KCl, 10 mM; sodium azide, 10 mM; ATP, 0.2 mM; potassium phosphate, 10 mM; pH 7.2 and Cyt *c* 50 µM in both the cuvettes of the spectrophotometer. The reaction was initiated (A) by the addition of succinate (10 mM) to the sample cuvette. The rate of reaction was monitored at 550 nm. In the next step of assay (B), the mitochondria were first osmotically ruptured by adding the mitochondrial sample directly to 0.225 ml of water (half of the reaction volume) in both cuvettes. The sample was mixed for 1 min and then 0.225 ml of twofold concentrated assay medium was added. The reaction was initiated and monitored as described before. Percent intactness was calculated as $(1\text{-A/B}) \times 100$. For all experiments, 75–80% intact mitochondria were used.

For the measurements of ATP translocation across the mitochondrial membrane and for the determination of the K_m for ADP of the translocator, a coupled enzyme assay using hexokinase and glucose-6-phosphate dehydrogenase was used as described earlier [6, 8]. For the ATP export experiment, the same assay mixture was used. The reaction was initiated by the addition of ADP (0.1 mM) and NADP (0.3 mM). The final levels of NADPH resulting from these reactions were measured spectrophotometrically at 340 nm (NADPH $E_{340} = 6.22 \times 10^{-3} \times \text{mol}^{-1} \times \text{cm}^{-1}$) and directly correlated to the amount of ATP exported. Using the coupled assay, the K_m of the ATP/ADP translocator was determined by measuring the ATP export rates in the presence of 0.3–1.2 mM ADP. The rate of the reaction was monitored and a double reciprocal graph was plotted. The K_m value was interpreted as the intercept on the X-axis of the graph. All the reactions mentioned above were carried out at 25 °C.

The K_m for ATP of the ATP-ADP translocator was also determined using [U-^{14}C]ATP as tracer as described previously [6]. The assay mixture was prepared by mixing 0.5 ml of the mitochondrial extract (depleted of endogenous nucleotides) and 0.49 ml of the incubation mix (adenosine 3′-phosphate, 10 mM; oligomycin, 120 µg/ml; EDTA, 0.5 mM; mannitol, 0.4 M, triethanolamine, KOH, pH 7.5, 20 mM). The control reactions included atractyloside (10 µM) prior to addition of unlabelled ATP and [U-^{14}C]ATP. In the experimental reaction the atractyloside (10 µM) was added after the addition of a mixture of the unlabelled ATP and the [U-^{14}C]ATP. The concentration of unlabelled ATP was varied from 10–50 µM and to each reaction tube was added 37 kBq (1.78 nmol) [U-^{14}C]ATP. To calculate ATP exported, 100 µl of the sample (from a total volume of 1.5 ml) was dried in a scintillation vial on a piece of Whatman 3 MM filter paper at 50 °C and to each scintillation vial 10 ml Bray's scintillation fluid was added. Counting was done in a LKB 1215 Rack Beta Liquid Scintillation Counter for each control and experimental vial for both A and B lines. The amount of ATP binding to the translocator was back calculated to pmol of ATP after plotting the values of ATP concentration (cpm) against substrate concentration (unlabelled ATP concentration). Values were obtained from this plot after normalization and the double reciprocal graph was plotted to determine the K_m for ATP.

Protein content was determined using the method of Peterson [9] and the total Cyt aa_3 content was determined spectrophotometrically [10] or by using the millimolar extinction coefficient of Cyt aa_3 at 550 nm (Cyt aa_3 $E_{550} = 19 \times 10^{-3} \times \text{mol}^{-1} \times \text{cm}^{-1}$) [11].

Results

ADP can enter intact mitochondria through the ADP-ATP translocator and be phosphorylated to ATP. The ATP so produced can then move out *via* the translocator and can be estimated by the coupled enzymatic assay.

Fig. 1 shows the efficiency of the ATP export from the mitochondria in the two lines. The rate of ATP export in the absence of ADP was negligible when the mitochondria were intact and this was taken as a control reaction. This was expected since no ATP formation would take place in the absence of ADP. When the mitochondria were broken and ADP was present, ATP produced by oxidative phosphorylation in the mitochondria drives the formation of NADPH very rapidly. The NADPH formation was found to be linear for about 15 min. No difference

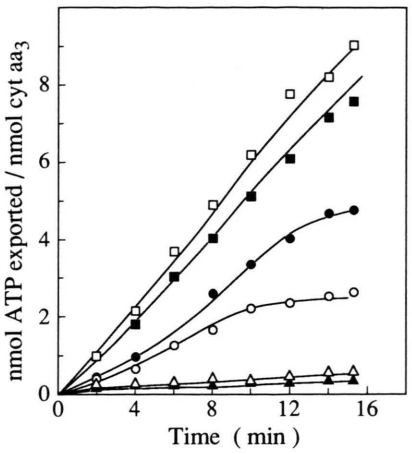

Fig. 1. ATP export from intact mitochondria in male sterile (MS) and male fertile (MF) sorghum lines: ATP export was monitored spectrophotometrically using the coupled glucose-6-phosphate dehydrogenase/hexokinase assay. The amount of NADPH formed (proportional to the amount of ATP exported *via* the ATP-ADP translocator) was monitored at 340 nm. MS ○--○, MF ●--● intact mitocondria with NADP and ADP; MS □--□, MF ×--× broken mitochondria with NADP and ADP; MS △--△, MF ▲--▲ intact mitochondria with NADP but without ADP.

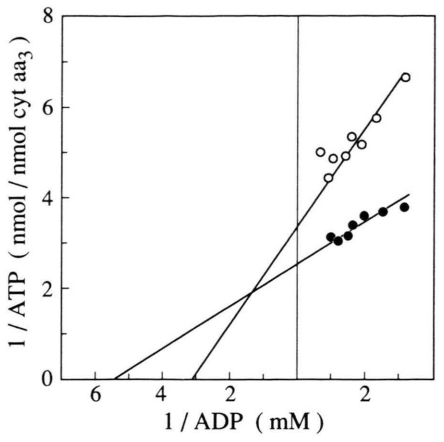

Fig. 2. Determination of the Michaelis-Menten constant (K_m) for ADP of the ADP-ATP translocator in mitochondria from the male sterile (MS) and male fertile (MF) lines. The amount of ATP exported was determined in terms of NADPH formed using the coupled assay as described in Fig. 1. The amount of NADPH formed in the presence of varying concentrations of ADP was monitored at 340 nm. K_m values were determined from Lineweaver-Burk plots. K_m in the MS line was found to be 326 μM as against 185 μM in the MF line. MS ○--○, MF ●--●.

was seen in the reduction of $NADP^+$ between the two lines. This served as the positive control for the reaction system. The export of ATP from the intact mitochondria was more efficient in the male fertile line as compared to the male sterile line in the presence of ADP. The result of this experiment, however, did not indicate any specific reason for this low level of ATP export (*i.e.* change in K_m or V_{max}) in the sterile line. It simply indicated lack of efficiency of the ATP-ADP translocator.

To investigate this further, the K_m value of the ATP-ADP translocator for ADP was determined in the mitochondria of male sterile and male fertile line. The K_m of the ATP-ADP translocator for ADP in the sterile line mitochondria was found to be 326 μM whereas for the fertile line mitochondria it was 185 μM (Fig. 2). The higher affinity of the translocator for ADP together with a higher V_{max} in the fertile line explains the high rate of ATP export seen in the case of fertile line.

Using the radiolabel method to determine the K_m of the translocator for ATP, it was found that in the sterile line the K_m value was twice (111 μM) that found for the fertile line (57 μM) as seen from Fig. 3.

It is, therefore, evident that the translocator from the male sterile line has a lower affinity for both ATP or ADP as compared to the translocator from the fertile line. Interestingly there was no differ-

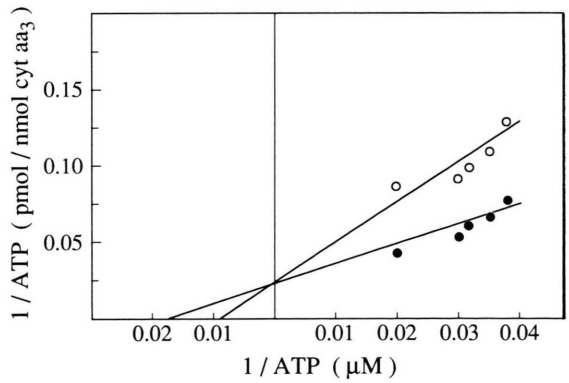

Fig. 3. Determination of the Michaelis-Menten constant for ATP of the ADP-ATP translocator in mitochondria from male sterile (MS) and male fertile (MF) plants. The K_m value was determined using the radio-labelling method by plotting the double-reciprocal graph (1/ATP *vs.* 1/ATP). The K_m in the MS line was found to be 111 μM as against 57 μM in the MF line. MS ○--○, MF ●--●.

ence in the V_{max} of ATP transport between the two lines.

Discussion

In our earlier studies we have shown that the rates of electron transport and ATP synthesis are reduced in the male sterile line. In the present study we have compared the translocation of ATP across the mitochondrial membrane in the male sterile and fertile lines. It was observed that mitochondria of the male sterile line are less efficient in ATP translocation and that this may be due to the lower affinity of the ATP/ADP translocator to the translocated nucleotides. These results are similar to those reported by Liu *et al.* [6] for petunia. They showed that the translocation of ATP in the fertile line of petunia was more efficient than in the sterile line. It was further shown that the K_m for ADP in the case of the fertile line was lower, together with a higher V_{max} as compared to the male sterile line. However, they found that although the K_m for ATP in the fertile line was lower than in the sterile line, the V_{max} in both the lines was not different. Considering the fact that ATP production in male sterile mitochondria of Sorghum (2219A) is lower and that whatever ATP is produced is not even translocated efficiently, we propose that the lower supply of ATP to the rapidly multiplying cells during microsporogenesis may result in male sterility.

The requirement of ATP during anther development is high and this necessitates faster multiplication of the mitochondria. It has, for example, been shown that in maize the number of mitochondria in anther cells during microsporogenesis increases by 20–30-fold [12]. Further, it has been shown that poor supply of ATP to rapidly dividing cells during microsporogenesis results in the degeneration of the tissue [4]. More recently, male sterility due to degeneration of tapetal cells has been observed [13]. It thus appears that low ATP supply could cause tapetal cell degeneration leading to male sterility.

The translocator protein is encoded by the nuclear genome, however, other components responsible for the total assembly of the ATP/ADP translocator in the mitochondrial membrane are encoded by the mitochondrial genome [14]. If the complementary components (some encoded by the nuclear genome and others by the mitochondrial genome) are not entirely compatible, the efficiency of the complete assembly could be affected. In the present study one could propose that translocator assembly is inefficient because the complementary components are only partially compatible.

The reciprocal control of gene expression between nucleus and organelle has been well demonstrated. In yeast it has been shown that the mitochondrial gene expression is regulated by the nuclear genome [15]. Such a regulation in incompatible cytoplasm and nucleus, a characteristic feature of CMS, could result in either inadequate production of a given component of the whole assembly or production of a faulty component. In either case the biochemical function of the assembly as a part of the entire energy transduction system could be adversely affected resulting in male sterility. This aspect in CMS has not yet been investigated but is worthy of attention by future research.

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