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Comparative analysis of mitochondrial and amyloplast adenylate translocators

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Structurally intact and metabolically competent mitochondria isolated from liquid-culture cells of sycamore (*Acer pseudoplatanus* L.) were shown to incorporate ADPglucose. Employing the double silicone oil layer filtering centrifugation method, we examined the kinetic properties of the uptake of various adenylates as well as the inhibitory effects exerted by carboxyatractyloside, atractyloside and bongkrekic acid, known specific inhibitors of the mitochondrial adenylate translocator. Immunoblot patterns of peptides derived from the partial proteolytic digestion of the mitochondrial and plastid adenylate translocators were shown to be essentially the same. We conclude that the molecular entities engaged in the adenylate transport system operating in two different organelles, mitochondria and amyloplasts, are very similar.

ADPglucose; Amyloplast; Mitochodrion; Silicone oil layer filtering centrifugation

1. INTRODUCTION

The adenylate (ADP/ATP) translocator is the major polypeptide component of the mitochondrial inner envelope membranes [1,2]. Its biochemical properties as well as the nuclear gene encoding the protein molecule have been thoroughly studied, using *Neurospora crassa* [3,4] and yeast [5,6].

We have detected an adenylate translocator in the envelope membranes of sycamore amyloplasts (*Acer pseudoplatanus* L.) using the specific antisera raised against the mitochondrial adenylate translocator of *N. crassa* [7]. Subsequently we have examined the kinetic properties of the adenylate translocating system present in plastids, and found that there is a direct transport of ADPG across the envelope membranes which is tightly linked to the starch biosynthesis catalyzed by starch synthase (ADPG: 1,4-α-glucan transglucosylase) [8]. In a further attempt to study structural and functional resemblances of the adenylate translocator in the two different types of organelle, we have analyzed its kinetic properties in sycamore mitochondria and compared with those of amyloplasts.

Abbreviations: ADPG, ADPglucose; At, atractyloside; BKA, bongkrekic acid; CAT, carboxyatractyloside.

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2. EXPERIMENTAL

2.1. *Reagents*

ATP, ADP, AMP, ADPG, At and CAT were purchased from Sigma (USA). Radiochemicals ([^{3}H]ATP, [^{3}H]ADP, [^{3}H]AMP and ADP-[^{14}C]G were purchased from Amersham (UK). Wackersilicone AR 20 Pharmas from Wacker-Chemie (Germany) and SH 704 from Toray Silicone (Osaka) were used for the transport experiments. The polyclonal antisera against *N. crassa* ADP/ATP translocator and BKA were kindly donated by Drs. W. Neupert and M. Klingenberg (Univ. of Munchen, Germany), respectively. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories (USA).

2.2. *Cell culture of sycamore and isolation of mitochondria and amyloplasts*

The liquid culture method of sycamore (*Acer pseudoplatanus* L.) was as described previously [9]. The isolation of intact amyloplasts and mitochondria was based on the methods as reported previously [8,10]. Mitochondria (Cyt *c* oxidase activity: 16.2 ± 4.7 U/ml and protein content: 4.2 ± 0.7 mg/ml, $n = 4$) and amyloplasts (6PGDH specific activity: 3.2 ± 7.9 U/ml and protein content: 12.7 ± 2.31 mg/ml, $n = 5$) were resuspended in sampling buffer containing 25 mM HEPES (pH 7.5); 20 mM KCl; 1 mM EDTA and 0.45 M mannitol ($\varrho = 1.031$ g/ml).

2.3. *Assay of adenylate translocation in mitochondria*

Unless otherwise specifically indicated, transport experiments were performed using Bio-Rik 0.4 ml plastic tubes from Bio-Plastics Co., USA. A conventional single silicone oil layer centrifugation technique was employed to assay the time-course uptake of adenylates in mitochondria [11]. Seventy-five microliter of silicone oil ($\varrho = 1.057$ g/ml) was layered onto 100 μl of pelleting layer (0.4 M sucrose in 10% $HClO_4$ ($\varrho = 1.064$ g/ml at 4°C)). Mitochondria preincubated for 30 min with 1 mM ATP before transport experiments were separately incubated in Eppendorf tubes at 4°C with the radiolabelled adenylate (200 μM). Subsequently, 40 μl of the mitochondrial sample (ca. 160 μg protein) were layered onto the silicone layer in each assay and tubes were centrifuged at $10\,000 \times g$ for 1 min in a Beckman microfuge B (USA). The quantity of adenylates transported was calculated as a function of the radioactivities recovered in the pelleting layer and concentrations of adenylates in the mitochondrial matrix were calculated

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after estimating the matrix volume (H_2O-permeable space minus intermembrane space (see below)).

For the kinetic studies of adenylate uptake, the double silicone oil layer centrifugation technique was employed throughout this investigation using a refrigerated TOMY MRX-151 centrifuge (Tokyo) (tubes were centrifuged at 10 181 × g, temperature set at 4°C and incubation time 1.2 s [8]. In this technique, incubation medium (60 μl of 25 mM HEPES (pH 7.5), 1 mM EDTA, 20 mM KCl, 0.35 M mannitol and 0.1 M sucrose, $\varrho = 1.041$) containing labelled adenylates of various concentrations as indicated in Fig. 2, with or without CAT (20 μM), BKA (35 μM) and At (35 μM), was sandwiched between two layers of silicone (light and heavy, $\varrho = 1.003$ and 1.057 g/ml, respectively). Incubation starts during centrifugation when mitochondria traverse the incubation medium for a short period (incubation time) depending on the centrifugal force. In experiments testing the inhibitory effect of BKA on the adenylate uptake, mitochondria were preincubated with sampling buffer (see above) which was adjusted to pH 6.5 [12,13].

Since the intermembrane space existing between the inner and outer membranes is accessible to every adenylate tested, it is essential to determine the real values of the adenylate transport mediated by the translocator located in the inner membrane which were obtained after estimating the intermembrane space. Although the experimental details of the technique employed will be described elsewhere (manuscript in prep.), the essence of the procedure is as follows. By keeping the amount of radiolabelled adenylates constant in the incubation layer of the double silicone oil-loaded centrifugation tubes (see above), it is expected that the radioactivities recovered in the pelleting layer will decrease in parallel with the reduction of the specific radioactivities of the adenylates; the probability for recognition of labelled adenylates by the adenylate translocator becomes zero when [adenylate] = ∞, and under such conditions the volume of the intermembrane space can be determined as a function of the radioactivities recovered in the pelleting layer.

2.4. *Immunoblot analysis*

Electrophoresis of sycamore amyloplasts and mitochondria and subsequent immunoblots were performed as described previously [7]. In order to make certain that the results of the amyloplast immunoblots are not ascribable to contaminating mitochondria, various amounts of mitochondria applied to the gel were used as negative controls. Peptide characterization of adenylate translocator from the two organelle types was carried out by partial proteolytic digestion according to the method of Cleveland et al. [14]. The electrophoresed gels were subsequently challenged with the specific antisera raised against the ADP/ATP translocator isolated from *N. crassa*.

3. RESULTS

3.1. *ATP/ADP/AMP/ADPG transport by mitochondria*

The single silicone oil layer centrifugation technique [11] was employed to determine the time-course uptake of adenylates. The results presented in Fig. 1A show that ATP/ADP/AMP uptake by mitochondria is extremely rapid, reaching the steady state in less than 25 s which is agreeable with the observation of Pfaff et al. [15]. However, in contrast to the case of amyloplasts [8], different steady-state levels were obtained for each adenylate with the mitochondrial system. CAT (20 μM), a potent inhibitor of the adenylate transport by mitochondria [13], was shown to strongly inhibit the uptake of ATP and ADP (Fig. 1B). Furthermore, it was found that sycarmore mitochondria incorporate not only ATP, ADP and AMP [16] but also ADPG and this transport was markedly inhibited by CAT (20 μM) (Fig. 1C).

Kinetic analysis of the adenylate uptake by mitochondria was performed employing the double silicone oil layer filtering centrifugation technique which allows us to determine the initial rates of adenylate uptake before the steady state level has been reached, and results are summarized in Fig. 2.

Although ADPG can serve as the glucosyl donor for starch biosynthesis, since only ADP-[^{14}C]G is commercially available, we were unable to determine the kinetic parameters of ADPG uptake in amyloplasts [8]. In the present work, however, ADPG can be taken up without subsequent transglucosylation which permits us to per-

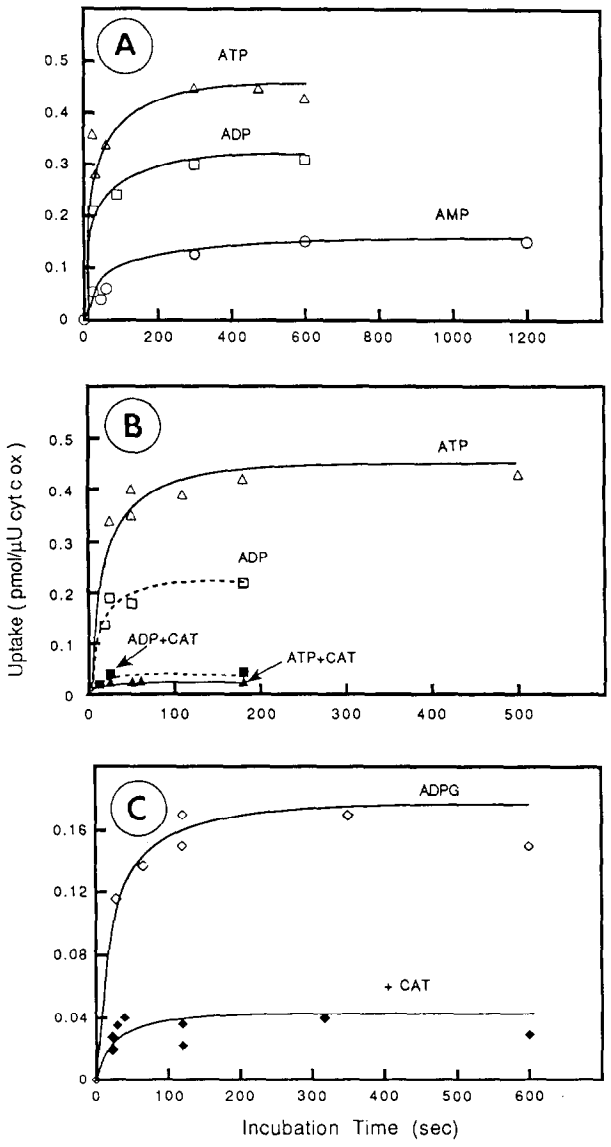

Fig. 1. (A) Time-course uptake of [^{3}H]ATP/ADP/AMP in mitochondria. (B) Inhibitory effect of CAT (20 μM) on ATP and ADP uptake in mitochondria. (C) Inhibitory effect of CAT (20 μM) on ADPG uptake in mitochondria.

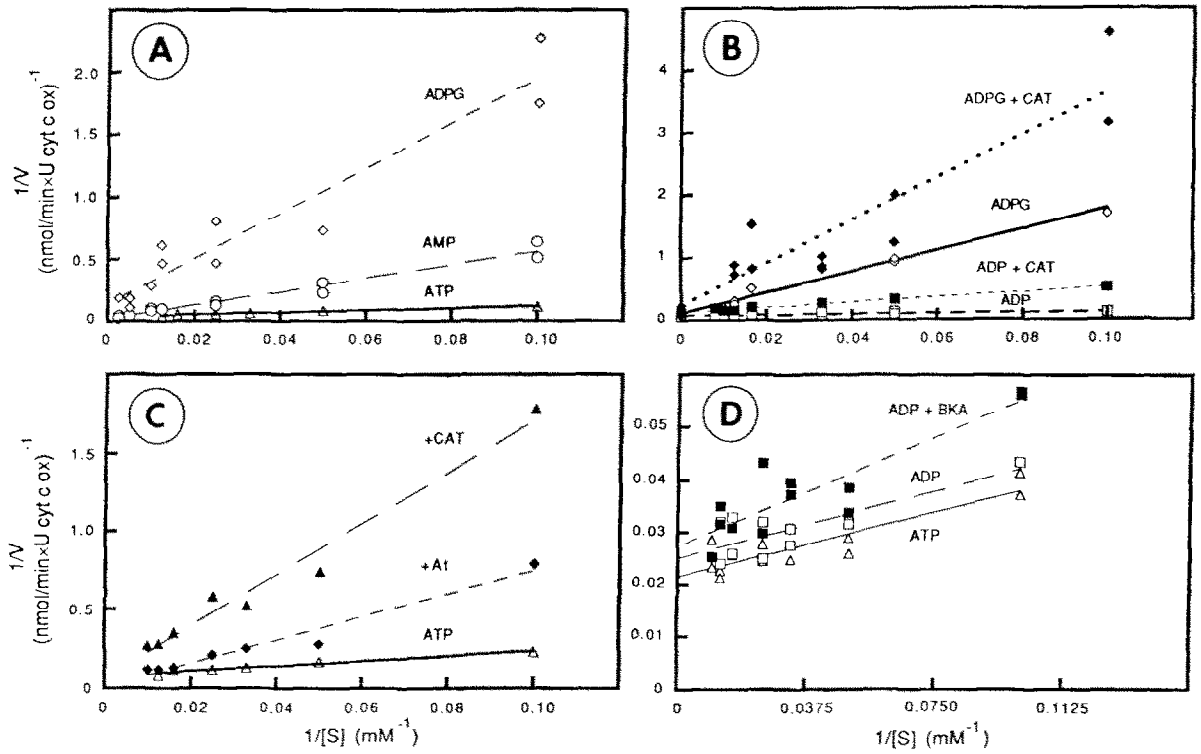

Fig. 2. Lineweaver-Burke plots of adenylates transport and inhibitory effect of CAT, At and BKA. (A) Uptake of ATP, AMP and ADPG. (B) Inhibitory effect of CAT (20 μM) on ADP and ADPG uptake. (C) Comparison of inhibitory effects of CAT (20 μM) and At (35 μM) on ATP uptake. (D) Inhibitory effect of BKA on ADP uptake. In Figs. 1 and 2 open symbols are used for ATP (△), ADP (□), AMP (○) and ADPG (◇) and effects of inhibitors are shown by closed symbols.

form the kinetic analysis of ADPG uptake in mitochondria. Fig. 2A,B shows that the results obtained were different from the case of plastids in which the affinities for different adenylates were nearly the same [8]. Affinities of mitochondrial adenylate translocator determined by triplicate experiments for ATP (K_m = 7.8, 11.2, 19.8 μM) and ADP (K_m = 6.8, 8.2, 14.7 μM) were markedly larger compared to those of AMP (K_m = 155, 187, 253 μM) and ADPG (K_m = 163, 159, 223 μM). CAT (20 μM) exhibited a potent inhibitory effect on the ATP/ADP/ADPG uptake (Fig. 2B,C). The inhibitory effect of ATP (10 μM) on the ADPG uptake by mitochondria is much larger than that observed in amyloplasts [8].

CAT, At and BKA are well-known inhibitors of the adenylate transport in rat liver mitochondria [13]. However, in agreement with previous reports showing the weak inhibitory effect of At and BKA in the adenylate uptake by plant mitochondria [13,17,18], inhibition by these reagents (35 μM) in the sycamore mitochondria is much smaller than that of CAT (Fig. 2C,D). Similar results have been observed in amyloplasts [8].

3.2. *Immunoanalysis of adenylate translocator of amyloplasts and mitochondria*

The presence of a 32 kDa adenylate translocator was first proposed from the immunoblot analysis of the amyloplast inner envelope membranes employing the antisera against the mitochondrial adenylate translocator from *N. crassa* [7]. However, we cannot totally rule out a possible cross-contamination of amyloplast envelope preparations with mitochondria in these previous experiments [19] and we have carefully repeated analyses to prove that the envelope membranes of sycamore amyloplasts indeed contain their own 32 kDa translocator polypeptide. Fig. 3 shows the presence of two polypeptides of ca. 30 kDa in the immunoblots when mitochondrial membranes containing Cyt *c* oxidase activities greater than 50 mU were loaded (lanes 1–3). However, a mitochondrial sample containing 1077 μU of Cyt *c* oxidase (0.093 μg protein) did not yield positive bands (lane 6), while amyloplasts (34 μg protein) containing as little as 76 μU of Cyt *c* oxidase gave a clear signal (lane 7). Results thus indicate that amyloplasts contain approximately two orders of magnitude less adenylate translocator when compared to mitochondria.

In a further attempt to compare the mitochondrial and amyloplastic adenylate translocators, limited digestion of both polypeptides by protease V8 was carried out. Electrophoreses digests were subsequently applied to the immunoblot analysis using antisera against the ADP/ATP-translocator from *N. crassa*. A careful

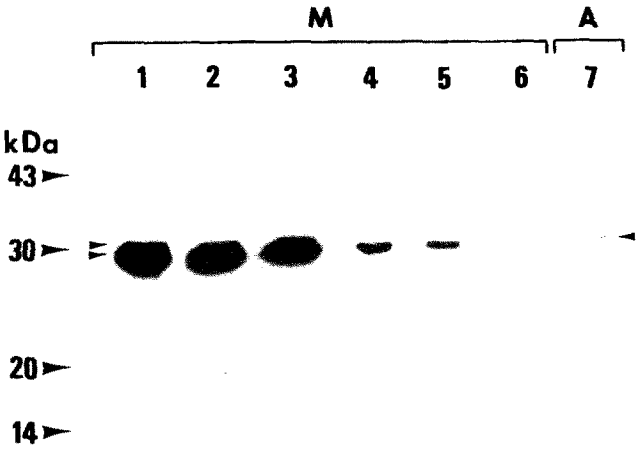

Fig. 3. Immunoblot analysis of mitochondria (M) and amyloplast (A) using antisera raised against mitochondrial ADP/ATP-translocator (*N. crassa*). Various amounts of mitochondria were loaded in decreasing order as measured by both Cyt *c* oxidase activities and protein content; Lane 1: 209 mU, 18.95 μg; lane 2: 104.5 mU, 9.47 μg; lane 3: 52.2 mU, 4.73 μg; lane 4: 8.6 mU, 0.75 μg; lane 5: 4.3 mU, 0.375 μg; lane 6: 1077 μU, 0.093 μg. lane 7: 34 μg of amyloplasts containing 76 μU of Cyt *c* oxidase.

scrutiny of the immunoblot presented in Fig. 4 corroborates a very close similarity between the band patterns of lanes 3 and 4, suggesting the likely identical primary structures in the peptide molecules derived from the two different organelles. It should be stressed that in conjunction with the results of Fig. 3, the peptide pattern shown in lane 4 is unlikely to be due to mitochondrial contamination.

4. DISCUSSION

The targeting of proteins into different organelles and subsequent assemblage are of great interest and importance from the standpoint of biological functions of organelles as well as for elucidating the molecular mechanism(s) underlying their biogenesis. It has been well established that the adenylate (ADP/ATP) translocator is an important molecular entity for the ATP synthesizing machinery existing in the mitochondria and for the energy compartmentation in the eukaryotic cells [1,2].

Since it has been reported that the precursor of the adenylate translocator molecule is not proteolytically processed and a membrane potential is required during the step of its transfer into mitochondria [20,21], we can predict that any organelle enclosed by energized membranes and requiring a continuous flow of energy (adenylates) from the cytosol for its own metabolic activities will entail an analogous targeting mechanism as that operating in mitochondria. In this context, the results obtained in the present investigation further strengthen our view that there exist closely related protein molecules engaged in the adenylate transport in two different plant organelles. Amyloplasts are metabolically active plastids but they lack the efficient ATP-generating machineries which are needed to sustain their own metabolism. It is thus natural to conceive that there operates an active flux of adenylates between the cytosol and the stromal matrix. It is also likely that chloroplasts, another plastid type, have an adenylate translocator which permits the uptake of ATP at night [22,23] and that of ADP serving as the P-acceptor in photophosphorylation [24]. Our recent experiments indeed support these previous presumptions (manuscript in prep.).

To our knowledge this is the first report showing ADPG uptake in mitochondira. Although the apparent affinities for ADPG and AMP determined are markedly smaller than that observed with amyloplasts, presumably because interactions between translocator molecules and the membranous environments are distinguishable in each respective organelle, the results obtained in the present investigation support our view that the mitochondrial type adenylate translocator in the plastid envelopes fulfils a crucial role in starch biosynthesis.

There has now been an ample amount of evidence showing that there exist multiple genes for the mitochondrial ADP/ATP translocator protein in different species [25–27]. The presence of two mitochondrial translocator proteins of ca. 30 kDa detectable in

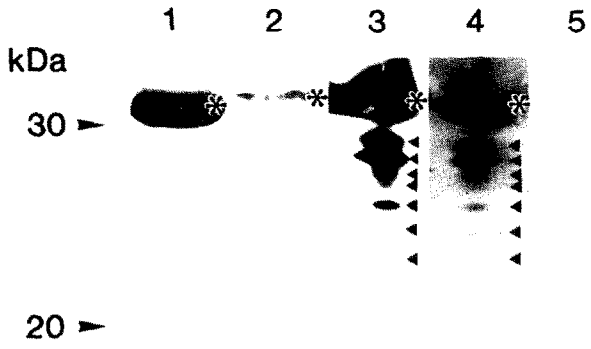

Fig. 4. Immunoblot analysis of proteolytic (*Staphylococcus aureus* protease V8) digests of mitochondria and amyloplasts using antisera against mitochondrial adenylate translocator from *N. crassa*. Lane 1: mitochondria (250 μg), no protease treatment; lane 2: amyloplast (500 μg), no protease treatment; lane 3: mitochondria (250 μg) + protease (V8) treatment; lane 4: amyloplast (500 μg) + protease (V8) treatment; lane 5: control (protease V8). Since the amount of translocator in amyloplasts is much smaller compared to mitochondria time exposure to develop the picture of lane 4 was longer than for other lanes.

the immunoblot (cf. Fig. 3) likely represents products encoded by multiple genes in the sycamore cells. A question arises as to whether or not the same unclear gene encodes for the translocator existing in the plastid envelopes. Research along this line is now in progress.

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