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Nucleotides metabolism in the chloroplast thylakoid lumen – evidence for a trans-thylakoid nucleotide transporter

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Introduction

The chloroplast stroma is the main compartment for nucleotide synthesis and utilization during photosynthesis, but the presence of nucleotides in the thylakoid lumen is largely unknown. However, there are indications for lumenal chaperones (Schlicher and Soll, 1996) and immunophilins (Fulgosi et al., 1998) as well as for nucleotide binding to lumenal proteins (Gal et al., 1996 and Spetea et al., these Proceedings *S8-005*). The lumen has previously been tested for the presence of ATP without any conclusive results (Kieselbach et al., 1998). Moreover, there are no reports on nucleotide transport across the thylakoid membrane. Therefore, we have used different biochemical approaches combined with database search to analyze the presence of activities involving nucleotides in the thylakoid lumen as well as the presence of a nucleotide translocator in the thylakoid membrane.

Materials and methods

Thylakoid membranes were isolated from spinach and resuspended as described in Spetea et al., these Proceedings *S8-005*. Thylakoid lumen was isolated from thylakoid membranes essentially according to Kieselbach et al., 1998.

Photolabeling with [γ-^{32}P]8-N_3ATP (19.8 Ci/mmol, ICN Pharmaceuticals) of dark-control or pre-illuminated thylakoids was performed as described for [α-^{32}P]8-N_3GTP (Spetea et al., these Proceedings *S8-005)*.

Nucleoside diphosphate kinase (NDPK) activity was measured based on the ability to transfer [^{32}P]phosphate from [γ-^{32}P]NTP (ATP, GTP) (3000 Ci/mmol, Amersham) to a nucleoside diphosphate (GDP, ADP). The reaction products were separated by thin-layer chromatography (TLC) on PEI-cellulose plates with 0.75 M KH_2PO_4 (pH 3.65). The resulting spots were visualized by phosphoimaging and quantified.

SDS-PAGE and western blotting were performed as previously described (Spetea et al., 1999). The photolabeled proteins were detected by phosphoimaging. The antibody raised against the beef heart mitochondrial ATP/ADP translocator, was a kind gift from Dr. J. Houstek. The NDPK antibody raised against the pea stromal enzyme was kindly provided by Dr. J. Soll.

Several protein databases available on the Internet were employed. Prediction for chloroplast and lumenal transit peptides were performed using TargetP and PSORT.

Results

Photoaffinity labeling with [γ-^{32}P]8-N_3ATP and immunological detection of a trans-thylakoid nucleotide transporter. In Spetea et al., these Proceedings *S8-005*, a 36.5 kDa protein was detected with [α-^{32}P]8-N_3GTP in dark-control thylakoids and its labeling was enhanced by pre-illumination. The lumenal 33 kDa subunit of photosystem II complex was also photolabeled but only in pre-illuminated thylakoids. **Fig.1*A*** shows the detection of the 36.5 kDa protein with [γ-^{32}P]8-N_3ATP in dark-control and pre-illuminated thylakoids. The photolabeling was 3-4 fold stimulated by light but not affected by DCMU. No labeling of the 33 kDa PSII subunit was detected, indicating that this protein does not bind ATP but GTP (see also Spetea et al., these Proceedings *S8-005*). Labeling of thylakoid subfractions indicated the location of the 36.5 kDa band mainly to the stroma-exposed membranes (not shown).

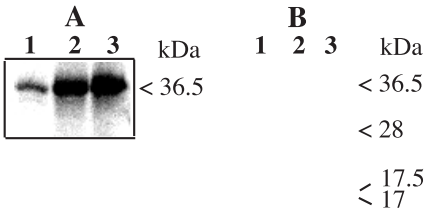

Fig. 1. Identification of a 36.5 kDa trans-thylakoid nucleotide transporter. *A*, Dark-control (*lane 1*) or pre-illuminated thylakoids (*lanes 2-3*) were photolabeled with 5 μM [γ-^{32}P]8-N_3ATP. 50 μM DCMU was added to the samples in *lane 3* at the onset of illumination. *B*, Thylakoid (*lane 1*) and envelope (*lane 2*) membranes were subjected to SDS-PAGE and western blotting with an antibody against the beef mitochondrial translocator. In *lane 3* is shown the cross-reaction of this antibody with beef mitochondria.

A well-characterized ATP transporter is the one from beef heart mitochondria (AAC). It has a calculated M.W. of 32 kDa, functions as a dimer and has six predicted transmembrane helices (Winkler and Neuhaus, 1999). In western blotting experiments performed with an antibody raised against the AAC protein, two bands with M_r of 28 and 17.5 kDa were detected in beef mitochondria, corresponding to the intact protein and a known degradation product, respectively (**Fig. 1*B*** *lane 3*). When this antibody was applied to thylakoids, two bands (36.5 and 17 kDa) were detected (*lane 1*) while no immuno-reaction was observed in the chloroplast envelope (*lane 2*). Western blots corresponding to the phosphoimaged gel presented in **Fig. 1*A*** showed that the 36.5 kDa labeled band was the one cross-reacting with the antibody (not shown). The 17 kDa band which was also found to be labeled in chloroplasts is presumably, in analogy with the mitochondrial 17.5 kDa band, a proteolytic fragment of the intact protein.

The calculated K_m values for the interaction of ATP with the putative translocator revealed two types of binding sites. In pre-illuminated thylakoids the binding of ATP is much tighter (K_m=7.7 μM) than in the samples kept in the dark (K_m=225 μM). Competition experiments in pre-illuminated thylakoids were carried out with 7 μM [γ-^{32}P]8-N_3ATP and 200 μM nucleotides. ATP, CTP and UTP prevented labeling to similar extents (65 %) whereas GTP inhibited by 50 %. These results indicate that all nucleoside triphosphates can be substrates for transport across the membrane.

Sequence homology search combined with prediction for chloroplast transit peptides indicate the presence of one candidate for a trans-thylakoid nucleotide transporter in *Arabidopsis thaliana* (EMBL: AL161946) with M.W. of 45.1 and 38.4 kDa for the precursor and mature forms, respectively. This candidate contains motifs for the mitochondrial carrier protein (PROSITE PS00215). It shows 45 % similarity to the AAC protein and 52 % similarity to a peroxisomal Ca^{2+}-dependent solute carrier. Hydropathy analysis revealed six putative transmembrane helices as for the AAC protein, and an N-terminal extension exposed to the lumenal side of the membrane. We suggest that the *Arabidopsis* homologue is the detected translocator in spinach thylakoids and, is therefore designated as the thylakoid nucleotide transporter (TNC).

Detection and characterization of nucleoside diphosphate kinase activity in the thylakoid lumen. ATP is the common form of energy in the cell metabolism. However, a number of processes require other nucleoside triphosphates, *e.g.* GTP. Nucleoside diphosphate kinase (NDPK, E.C.2.7.4.6) is a ubiquitous enzyme, which catalyzes the transfer of the γ-phosphate of 5'-triphosphate nucleosides to 5'-diphosphate nucleosides. NDPK genes have been cloned from a variety of eukaryotes including plants. Previously, NDPK activity has been measured in the cytosol (NDPKI) and the chloroplast (NDPKII and III), and the corresponding enzymes have been purified from spinach (Zhang et al., 1995, Yang and Lamppa, 1996) and pea (Lübeck and Soll, 1995).

In the present study, production of GTP was detected in the isolated preparation of thylakoid lumen incubated with [γ-^{32}P]ATP in the presence of GDP (**Fig. 2*A***), demonstrating for the first time the existence of NDPK activity in this chloroplast compartment. The optimum pH for this NDPK activity was 6.0 as compared to 7.5 for the isoforms isolated from the cytosol or the chloroplast (Zhang et al., 1995), which would be consistent with its lumenal location. The present NDPK activity is stimulated by the light-dependent events occurring in the thylakoid membrane. The determined affinity constant (K_m) for GDP was 27.4 μM, which does not correspond to any of the ones previously reported for the other NDPK isoforms (Zhang et al., 1995). Our present lumenal NDPK can also use [γ-^{32}P]GTP and ADP to produce [γ-^{32}P]ATP (K_m=89.05 μM). Its higher affinity for GDP suggests its main role in GTP synthesis and brings it closer to NDPKIII. This strongly suggests that NDPKIII is located in the lumen despite previous ambiguous interpretations on its location.

Western blot analysis with antibodies raised against the stromal NDPKII, which can cross-react with all NDPK isoforms, shows that NDPK is present in both stroma and thylakoid lumen (**Fig. 2*B***), but as two different proteins. The NDPKII migrates at 18.5 kDa (*lane 1)*. The lumenal NDPK migrates as a lower band of 17.2 kDa (*lane 2*), in analogy with the spinach NDPKIII (Zhang et al., 1995).

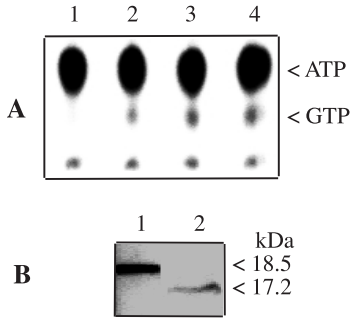

Fig. 2. *A*. Detection of NDPK activity in the thylakoid lumen. Thylakoid lumen (2 μg protein) was incubated with 200 nCi [γ-^{32}P]ATP 10 nmoles ATP and 10 nmoles GDP in a final volume of 10 μl for 0, 1, 3 and 7 min (lanes 1-4) at 35 ˚C. The reaction was stopped with 0.4 N HCOOH. The nucleotides were separated by TLC and the radioactive ones detected with the phosphoimager. *B.* Western blot with anti-NDPKII of stroma (*lane 1*) and thylakoid lumen (*lane 2*).

These biochemical data are consistent with the search in the *Arabidopsis* database. Only one NDPK precursor with potential chloroplast and thylakoid lumen transit peptides was found: NDPKIII (EMBL: AL049525), having a M.W. of 25.7 kDa while the mature form has a calculated M.W. of 17.1 kDa.

Discussion

In this work we report for the first time the detection of a nucleotide translocator in the chloroplast thylakoid membrane (designated TNC), confined to the stroma-exposed regions supplying the lumen with ATP. Previously, the isolation of an *Arabidopsis* cDNA clone encoding a 62 kDa membrane protein homologue to a bacterial ATP/ADP translocator was reported (Winkler and Neuhaus, 1999). This protein is localized in the chloroplast envelope and characterized as an ATP-importer into the inter-envelope space from the cytosol. It does not share significant sequence similarities with the mitochondrial AAC protein. Homology search with the AAC protein against *Arabidopsis* database indicated one candidate for a trans-

thylakoid nucleotide transporter, that is likely to correspond to the TNC protein characterized in this work.

The results presented here bring also for the first time evidence for NDPK activity in the thylakoid lumen of chloroplasts, which is likely to correspond to the spinach NDPKIII, suggesting the occurrence of nucleotide metabolism in the lumen.

Our present results summarized in **Fig. 3**, provide the following crucial implications: (*i*) The thylakoid membrane contains a nucleotide transporter (TNC) that is dependent upon electron transport activity; to study its mechanism with respect to activation and function will be a crucial task for future research; (*ii*) The thylakoid lumen contains nucleotides, and should be considered as a metabolicaly active compartment requiring energy; (*iii*) The involvement of GTP-induced signal transduction across the thylakoid membrane should also be elucidated.

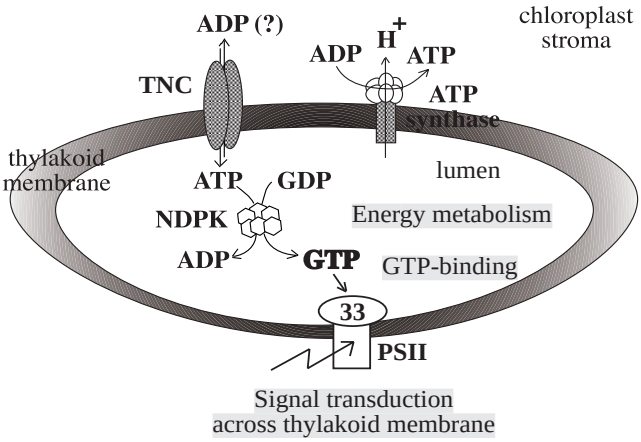

Fig. 3. Stromal ATP is transported by the TNC protein across thylakoid membrane into the lumen where NDPK converts it to GTP. Following photoinactivation of PSII, GTP binds to the 33 kDa subunit, and thus can influence various lumenal and membrane-bound effectors.

Acknowledgments

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