Plant mitochondrial nucleoside diphosphate kinase is attached to the membrane through interaction with the adenine nucleotide translocator

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Abstract This study shows that the plant mitochondrial nucleoside diphosphate kinase (mNDPK) localizes to both the intermembrane space and to the mitochondrial inner membrane. We show that mNDPK is very firmly attached to the membrane. Co-immunoprecipitation experiments identified the adenine nucleotide translocator as an interaction partner. This is the first report showing a direct association between these two proteins, although previous studies have shown metabolic cooperation between them. Possible consequences for mitochondrial energy metabolism are discussed.

Key words: Plant mitochondrion; Nucleoside diphosphate kinase; Adenine nucleotide translocator

1. Introduction

Nucleoside diphosphate kinases (NDPKs) are ubiquitous enzymes involved in equilibration of the cellular nucleosides triphosphate pools in most cellular compartments where they transfer the γ phosphate group from nucleoside triphosphates to nucleoside diphosphates. Besides this basic metabolic function, they are also involved in other vital processes such as control of cell proliferation [1], regulation of transcription [2,3] and protein phosphotransferase activity [4–6]. NDPKs have also been shown to interact with G-proteins [7], presumably in order to supply the GTP. In plants, phytochrome A response [8], UV-B light signaling [9], hormone [10,11] and heat shock response [12,13] are among the processes in which NDPK isoforms participate. It was recently shown that a plant NDPK interacts with two stress-activated MAP-kinases and is thereby involved in stress tolerance [14].

Many of the non-metabolic functions are mediated by protein–protein interactions between NDPKs and other proteins. These interactions have in some cases been shown to modulate the other proteins activity such as the chaperone function of hsp70 [15].

Struglics and Håkansson purified the first plant mitochondrial NDPK (mNDPK) isoform from pea and suggested an intermembrane space (IMS) localization [16]. This was later confirmed using a proteomics approach for mNDPK from potato and *Arabidopsis thaliana* by Sweetlove et al. [17]. The methods employed to prove that mNDPK is localized to the IMS do not exclude the presence of mNDPK in other submitochondrial compartments.

mNDPK, as many of the NDPKs, may be part of the cells response to external signals and this activity would probably involve protein–protein interactions. In order to evaluate any interactions found by methods such as pull-down and yeast-two hybrid systems, it is critical to know if the interacting protein resides in the same compartment. The aim of this study was to elucidate whether mNDPK exists in other submitochondrial compartments than the IMS and if so what potential interaction partners there might be.

2. Materials and methods

2.1. Plant material

Garden peas (*Pisum sativum* L. cv Oregon sugarpod) were grown on vermiculite at 20°C with a 12 h day. Mitochondria were isolated as in [18] with modifications according to Håkansson et al. [19].

2.2. Submitochondrial fractionations

Mitochondria were fractionated into membrane and soluble fractions by sonication. Mitochondria were diluted to a concentration of 2 mg ml^{-1} in 0.3 M sucrose, 50 mM Tris–HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF). They were sonicated four times for 30 s on ice in a Soniprep 150 MSE. Sonicated mitochondria were centrifuged for 45 min at 104 000 × *g* in order to separate membranes from the soluble fraction.

Mitoplasts were prepared from freshly isolated mitochondria taken up in 50 mM sucrose, 10 mM MOPS–KOH pH 6.7, 1 mM PMSF to a concentration of 10 mg mitochondria ml^{-1} and stirred on ice for 15 min. The osmotic strength was adjusted to 0.3 M sucrose and the suspension centrifuged for 15 min at 13 000 × *g*. The pellet represented the mitoplasts.

2.3. Mitoplast washes

Mitoplasts were taken up in freshly made washing agents as indicated in the legend to Fig. 2 and incubated for 10 min at room temperature. The mitoplasts were centrifuged at 13 000 × *g* in a microfuge at 4°C for 20 min. The final pellet was taken up in Electrophoresis Sample Buffer (Invitrogen).

2.4. Co-immunoprecipitation

The solubilization buffer used was 150 mM NaCl, 50 mM Tris–HCl pH 8.0, 0.25% IGEPAL [(octylphenoxy)-polyethoxyethanol], 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc; Mannheim-Boehringer). Samples were diluted to 450 μl in solublization buffer (typically 50 μg). The lysate was pre-cleared by the addition of 5 μl of pre-immunsera and 50 μl of pre-swelled protein A-Sepharose (Pharmacia) for 1 h at 4°C. The samples were centrifuged for 13 000 × *g* for 25 s and the supernatant transferred to a fresh tube.

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Abbreviations: ANT, adenine nucleotide translocator; IMS, intermembrane space; mNDPK, mitochondrial nucleoside diphosphate kinase; PMSF, phenylmethylsulfonyl fluoride

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The supernatant was further incubated with 5 μl antibody solution for 1 h followed by addition of 50 μl of protein A beads and a further 1 h incubation. The beads were collected and washed three times with 1 ml of solubilization buffer and once with 50 mM Tris–HCl pH 8.0. The final pellet was taken up in Electrophoresis Sample Buffer (Invitrogen), heated for 3 min at 95°C and the beads were collected. The supernatants were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.5. Electrophoresis

Samples were separated on NuPage Bis–Tris Gels (Invitrogen) in NuPage MOPS SDS buffer according to the instructions of the manufacturer (NuPage® Novex high-performance pre-cast gels Bis–Tris gel). SeeBlue® Plus2 pre-Stained protein standard (Invitrogen) was used as a molecular marker.

2.6. Western blot

Electrophoretic transfer of proteins to nitrocellulose membrane was performed according to the instructions of the manufacturer (Invitrogen). mNDPK antibodies [13] were used in a 1:6000 dilution, Adenine Nucleotide Translocator (ANT (FL298):sc-11433, Santa Cruz Biotechnology) antibodies 1:500, cytochrome *c* (clone 7H8.2C12, BD PharMingen) antibodies in a 1:1000 dilution. Immunodetections were performed using the Enhanced Chemiluminescence Plus Detection System (ECL, Amersham Pharmacia).

2.7. Mass spectrometry (MS) identification

Excised bands were digested in the gel with trypsin. The peptides were treated according to Eklund and Edquist [20]. This method is a modified version of that described by Wilm et al. [21]. To acquire peptide sequence data capillaries were inserted into a quadruple time-of-flight (QTOF)-MS instrument (Micromass Q-Tof, Micromass Ltd) with a nanospray ion source. The capillary voltage was set to 800–900 V and the cone voltage to 40 V. Argon was used as collision gas and the kinetic energy was set between 20 and 40 V. Peptides were sequenced using the BioLynx program of the MassLynx NT software package (version 3.4, Micromass Ltd).

3. Results

During the purification work by Struglics and Håkansson, it was shown that NDPK autophosphorylation activity could only be found in soluble mitochondrial fractions. The subsequent purification was therefore performed using the soluble mitochondrial fraction. Animal mitochondria contain NDPK activity both in the matrix and in the IMS. We wanted to determine if a proportion of the NDPK also is localized to the matrix in plants. In order to exclude any membrane association of mNDPK, mitochondrial membranes were separated from the soluble fraction by sonication and ultracentrifugation. Western blotting using antibodies produced against the

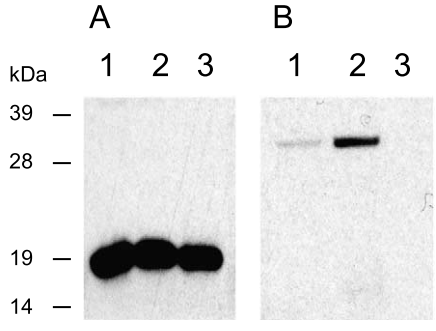

Fig. 1. mNDPK is both a soluble and a membrane-associated protein. Lane 1: mitochondria; lane 2, mitochondrial membrane fraction; lane 3, mitochondrial soluble fraction. Panel A was probed with NDPK antibody and panel B with ANT antibody. Molecular standard is indicated on the left.

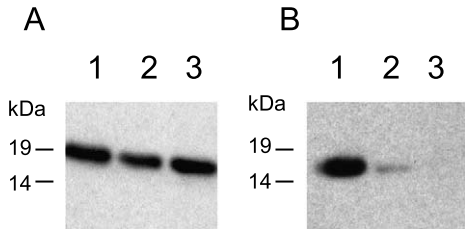

Fig. 2. Investigation of the strength of mNDPK membrane association. Lane 1 shows unwashed mitoplasts, lane 2 shows mitoplasts washed with 0.2% Triton X-100, and lane 3 shows mitoplasts washed with 200 mM Na_2CO_3. Panel A was probed with mNDPK antibody and panel B was probed with cytochrome *c* antibody. Molecular standard is indicated on the left.

C-terminus of the protein [13] was used to screen the distribution of mNDPK. In contrast to Struglics and Håkansson, we monitored the presence of the protein and not the activity. Fig. 1A shows that there is as much NDPK in the membrane as in the soluble fraction. No ANT could be found in the soluble fraction (Fig. 1B, lane 3) indicating low contamination of membranes in the soluble fraction. In order to study the nature of mNDPK association to the mitochondrial membrane, washes were performed. However, submitochondrial vesicles were formed upon sonication of mitochondria. These were in the inside-out orientation where proteins normally on the outside of the inner membrane are inside the vesicles and thereby protected from washes (data not shown). The membrane washes were therefore performed on so-called mitoplasts. Mitoplasts are mitochondria that have been treated to osmotic shock so that the outer membrane has ruptured leaving the outside of the mitochondrial inner membrane exposed. The results show that mNDPK is very firmly attached to the membrane (Fig. 2A). Washes that abolish the membrane association of the peripheral inner membrane protein cytochrome *c* (Fig. 2B) only reduce a small fraction of the membrane-attached mNDPK.

Immunoprecipitation was performed in order to identify putative membrane proteins that NDPK interacts with using mitochondrial membrane fractions and the mNDPK antibody

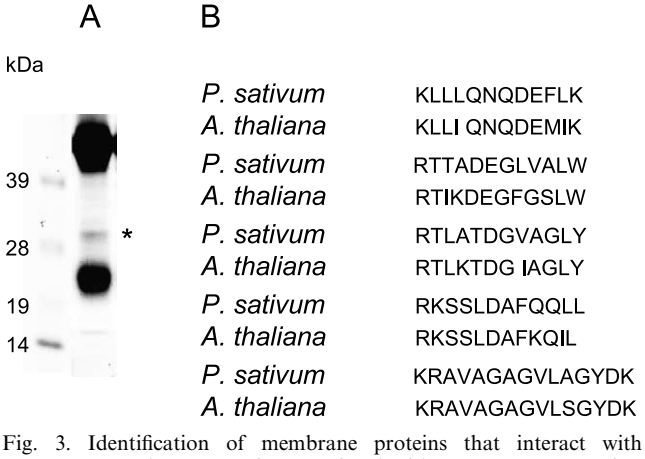

Fig. 3. Identification of membrane proteins that interact with mNDPK. Membrane proteins associated with mNDPK were co-immunoprecipitated using mNDPK antibodies. Panel A shows a colloidal Coomassie-stained gel. The marked band was cut out and used for peptide sequencing. Panel B shows the obtained peptide sequences aligned to the most similar *A. thaliana* sequence (accession number P31167). Molecular standard is indicated on the left.

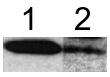

Fig. 4. Confirmation of interaction between mNDPK and the ANT by cross-immunoprecipitation. Western blot probed with the mNDPK antibody. Lane 1 shows mitochondrial membrane, and lane 2 shows a co-immunoprecipitation using the ANT antibody.

as described in Section 2.4. The immunoprecipitate was run on SDS–PAGE and the gel was stained. The two major bands most likely represent the heavy and light chains of the antibody (Fig. 3A). The marked band in Fig. 3A was cut out and in-gel digested followed by QTOF-MS analysis. The sequence is most similar to *A. thaliana* Adenylate translocator (accession number P31167). The peptide sequences cover 17% of the *Arabidopsis* protein and were found to be 87% identical, within the constraints of MS sequencing, thereby allowing us to conclude that the immunoprecipitated protein most likely corresponds to a pea ANT isoform. The interaction was confirmed by co-immunoprecipitation of mNDPK with a commercial antibody directed against the human ANT (Fig. 4, lane 2).

4. Discussion

NDPKs have been shown to interact with many protein partners with diverse cellular effects. It is therefore important to know which submitochondrial compartments that contain NDPK to be able to critically evaluate protein–protein interaction experiments. We and others have shown that mNDPK exists as a soluble entity in the IMS. In this paper we show that a substantial proportion, usually around 50%, is found attached to the mitochondrial membrane. Due to the strong interaction between NDPK and the mitochondrial membrane fraction it was impossible to exclude the possibility that there is a proportion also in the matrix. There is only one NDPK gene with predicted mitochondrial localization in the *Arabidopsis* genome. It is however possible that this gene product is targeted to multiple submitochondrial compartments, a mechanism that would merit further investigations from a protein targeting issue. Plant mNDPKs have a very long, 80 amino acids, presequence containing an intramitochondrial targeting sequence [22]. The mNDPK is most likely targeted to the matrix and then out again. It is possible that a proportion of mNDPK remains in the matrix during protein import.

The resistance of mNDPK to washes could also indicate localization to the contact points between the outer and inner mitochondrial membrane. The contact points are stable protein complexes that may protect mNDPK from detaching from the membrane. Models of the contact sites involve functional complexes of creatine kinase and hexokinase, the outer membrane porin and the inner membrane adenine nucleotide translocator [23]. The function of the NDPK–ANT interaction could be to locally decrease the ATP concentration on the IMS side of the inner membrane and thereby facilitate a higher rate of ATP export without building up an unfavorable ATP gradient over the membrane. Metabolic cooperation has been shown between NDPK and ATPsynthase by Roberts et al. [24], where it also was shown that plant mitochondria contains sufficient NDPK activity to regenerate nucleoside triphosphates at a rate higher than the maximum rate of respiration. Mitochondrial ATP is, at least in part, sequestered in a protein bound form that has been shown to be available to NDPK and Adenylate Kinase. This ATP can then be converted to ADP by these two enzymes and used to prime ATP-synthase. Ko et al. [25], working in a mammalian model system, have recently been able to isolate what they call an ATPsynthasome, a vesicle (cristae fraction) devoid of respiratory complexes but containing a complex of ATPsynthase, the ANT and the phosphate translocator [25]. It will be important to know whether NDPK exists in such cristae vesicles or not in plant and animals. The function of the interaction between NDPK and ANT could also be to, depending on the activity of the ANT, produce more or less of another nucleoside triphosphate e.g. GTP for a G-protein. It will be very interesting to see if the ANT that binds NDPK is the ANT in the contact points, the ANT in the ATPsynthasome or it is an ANT-NDPK complex by itself.

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References

[1] Cipollini, G., Berti, A., Fiore, L., Rainaldi, G., Basolo, F., Merlo, G., Bevilacqua, G. and Caligo, M.A. (1997) Int. J. Cancer 73, 297–302.
[2] Ji, L., Arcinas, M. and Boxer, L.M. (1995) J. Biol. Chem. 270, 13392–13398.
[3] Postel, E.H., Berberich, S.J., Flint, S.J. and Ferrone, C.A. (1993) Science 261, 478–480.
[4] Wagner, P.D. and Vu, N.D. (2000) J. Biol. Chem. 275, 35570–35576.
[5] Ogura, Y., Yoshida, Y., Yabe, N. and Hasunuma, K. (2001) J. Biol. Chem. 276, 21228–21234.
[6] Engel, M., Veron, M., Theisinger, B., Lacombe, M.L., Seib, T., Dooley, S. and Welter, C. (1995) Eur. J. Biochem. 234, 200–207.
[7] Cuello, F., Schulze, R.A., Heemeyer, F., Meyer, H.E., Lutz, S., Jakobs, K.H., Niroomand, F. and Wieland, T. (2003) J. Biol. Chem. 278, 7220–7226.
[8] Choi, G., Yi, H. and Lee, J. (1999) Nature 401, 610–613.
[9] Zimmermann, S., Baumann, A., Jaekel, K., Marbach, I., Engelberg, D. and Frohnmeyer, H. (1999) J. Biol. Chem. 274, 17017–17024.
[10] Novikova, G.V., Moshkov, I.E., Smith, A.R., Kulaeva, O.N., Hall, M.A. and Veron, M. (1999) Planta 208, 239–246.
[11] Nato, A., Mirshahi, A., Tichtinsky, G., Mirshahi, M., Faure, J.P., Lavergne, D., De Buyser, J., Jean, C., Ducreux, G. and Henry, Y. (1997) Plant Physiol. 113, 801–807.
[12] Moisyadi, S., Dharmasiri, S., Harrington, H.M. and Lukas, T.J. (1994) Plant Physiol. 104, 1401–1409.
[13] Escobar Galvis, M.L., Marttila, S., Håkansson, G., Forsberg, J. and Knorpp, C. (2001) Plant Physiol. 126, 69–77.
[14] Moon, H., Lee, B., Choi, G., Shin, D., Prasad, D.T., Lee, O., Kwak, S.S., Kim, D.H., Nam, J., Bahk, J., Hong, J.C., Lee, S.Y., Cho, M.J., Lim, C.O. and Yun, D.J. (2003) Proc. Natl. Acad. Sci. USA 100, 358–363.
[15] Leung, S.M. and Hightower, L.E. (1997) J. Biol. Chem. 31, 2607–2614.
[16] Struglics, A. and Håkansson, G. (1999) Eur. J. Biochem. 262, 765–773.
[17] Sweetlove, L.J., Mowday, B., Hebestreit, H.F., Leaver, C.J. and Millar, A.H. (2001) FEBS Lett. 508, 272–276.
[18] Boutry, M., Vassarotti, A., Ghislain, M., Douglas, M. and Goffeau, A. (1984) J. Biol. Chem. 259, 2840–2844.
[19] Håkansson, G., van der Mark, F., Bonnett, H. and Glimelius, K. (1988) Theor. Appl. Genet. 76, 431–437.

[20] Eklund, D.M. and Edqvist, J. (2003) Plant Physiol. 132, 1249–1259.
[21] Wilm, M., Neubauer, G. and Mann, M. (1996) Anal. Chem. 68, 527–533.
[22] Escobar Galvis, M.L., Håkansson, G., Alexciev, K. and Knorpp, C. (1999) Biochimie 81, 1089–1096.
[23] Ruck, A., Dolder, M., Wallimann, T. and Brdiczka, D. (1998) FEBS Lett. 426, 97–101.
[24] Roberts, J., Aubert, S., Gout, E., Bligny, R. and Douce, R. (1997) Plant Physiol. 113, 191–199.
[25] Ko, Y.H., Delannoy, M., Hullihen, J., Chiu, W. and Pedersen, P.L. (2003) J. Biol. Chem. 278, 12305–12309.