The Role of Nucleoside Diphosphate Kinase in Plant Mitochondria

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Cover: The first structure of plant NDPK3 from *Pisum sativum*. Ribbon representation of the trimer viewed down the 3-fold axis, showing three monomers.

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Babičce Ilzičce, Dědovi Zdenkovi
Mamce a Tátovi

Today was good
Today was fun
Tomorrow is another one

Dr. Seuss

Abstract

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Nucleoside Diphosphate Kinase (NDPK) catalyses the transfer of a phosphate from nucleoside triphosphates to a nucleoside diphosphate, is ubiquitously found in all organisms from bacteria to human. It was discovered that the genes *nm23* and *awd*, which encode NDPKs are involved in tumour metastasis and *Drosophila* development, respectively. Thus, NDPK isoforms have been suggested to have specific regulatory functions in addition to their catalytic activity. Plant NDPKs are also involved in a number of intracellular signalling events such as phytochrome A response, UV-B light signalling and heat shock response. The main focus in this thesis concerns the role of the plant (*Pisum sativum* L. cv Oregon sugarpod) mitochondrial NDPK3 isoform.

The NDPK3 is localized to both the intermembrane space and to the mitochondrial inner membrane. The membrane bound NDPK3 is firmly attached to the membrane through the ATP/ADP translocase. The ATP/ADP translocase and NDPK3 complex may be a part of the contact sites for channelling metabolites from mitochondria to cytosol and *vice versa*.

NDPK3 was shown to be dually targeted to both mitochondria and chloroplasts where the major amount of the protein is found in mitochondria. The protein was crystallized and the first X-ray structure of a plant NDPK is reported. In agreement with other eukaryotic NDPKs, the plant enzyme is a hexamer. Two conserved serine residues, S119 and S69 involved in serine autophosphorylation and oligomerization, respectively, was identified. We show that the Ser autophosphorylation depends on enzyme activity. The mutation of S69 to Ala decreased the enzymatic activity dramatically. Changes in the oligomeric pattern of S69A were observed. Thus, the S69 residue is important for the stabilization of the oligomeric state of NDPK3.

Adenylate Kinase was identified as an interacting partner of the IMS located NDPK3. The interaction modulates the activity of the enzymes where Adenylate Kinase stimulates NDPK3 and NDPK3 inhibits Adenylate Kinase with unchanged ADP production as an outcome. Cyclic AMP (cAMP) and calcium inhibit the activity of both NDPK3 and Adenylate Kinase. This is a novel regulatory relationship between cAMP and calcium signalling and nucleotide metabolism mediated by NDPK3 and Adenylate Kinase and their interaction.

Key words: NDPK, plant mitochondria Adenylate Kinase, ATP/ADP translocase, X-ray structure, cAMP, calcium.

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Abbreviations

List of selected abbreviations used in the text:

AK	Adenylate Kinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANT	Adenine nucleotide translocator
Ap5A	Di(Adenosine)Pentaphosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
dTDP	Deoxythymidine 5'-diphosphate
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
GA	Glutaraldehyde
GFP	Green fluorescent protein
IMP	Inner membrane protease
IMS	Inter membrane space
LDH	Lactate dehydrogenase
MPP	Mitochondrial processing peptidase
NADH	Nicotinamide adenine dinucleotide
NDPK	Nucleoside diphosphate kinase
NDPK:AK	Nucleoside diphosphate kinase:Adenylate Kinase complex
PEP	Phosphoenolpyruvate

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Appendix

This thesis is based on the following papers, which will be referred to as their Roman numerals**.**

I. Knorpp, C., Johansson, M. & Baird, A. M. 2003. Plant mitochondrial nucleoside diphosphate kinase is attached to the membrane through interaction with the adenine nucleotide translocator. *FEBS Letters 555*, 363-366.

II. Johansson, M., Mackenzie-Hose, A., Andersson, I. & Knorpp, C. 2004. Structure and mutational analysis of a plant mitochondrial nucleoside diphosphate kinase. Identification of residues involved in serine phosphorylation and oligomerization. *Plant Physiology 136*, 3034-3042.

III. Hammargren, J., Sundström, J., Johansson, M., Bergman, P. & Knorpp, C. 2006. On the phylogeny, expression and targeting of plant Nucleoside Diphosphate Kinases. *Physiologia Plantarum* Published online 23 August 2006. PPL 0794.

IV. Johansson, M., Uppsäll, E., Mackenzie, A. & Knorpp, C. 2006. The activity of Nucleoside Diphosphate Kinase and Adenylate Kinase are influenced by cAMP and calcium, as well as by their interaction. (Submitted).

Introduction

Mitochondria are energy providing organelles with two membranes, constantly fusing and dividing (Sesaki *et al.*, 2003). Mitochondria are believed to have been free-living bacteria that merged with a primitive cell. The mitochondrion contains its own genome and its own transcription and translation machinery. The vast majority of the mitochondrial proteins are, however, encoded in the nucleus, produced as precursor proteins in the cytosol and subsequently imported to the organelle. One of these proteins is Nucleoside diphosphate kinase (NDPK).

NDPKs have been characterized as a large family of conserved proteins that synthesize nucleoside triphosphates from nucleoside diphosphates (Lacombe *et al.*, 2000). One of the main functions of NDPKs is the maintenance of the intracellular nucleoside triphosphate pools. It has become recognized that as well as having the kinase activity, NDPK proteins have additional or different roles. This study concerns an NDPK isoform located in plant mitochondria that supports the ever-surprising functions of the protein. There is increasing evidence that NDPK is an important multifunctional protein involved in cell signalling and coordination of other proteins.

Mitochondria

When mitochondria were first discovered in the end of the 1800s, many scientists were surprised by how much they resembled bacteria. First 160 years later it was realized that mitochondria and chloroplasts have genes of their own. Mitochondria use their DNA, similar to the DNA of prokaryotes, to make their own proteins, and they duplicate themselves independently of the cell. Yet scientists still lacked the tools for finding out exactly what sort of DNA mitochondria and chloroplasts carried. There was a hypothesis suggesting that the mitochondrial and chloroplast genes had originated inside the nucleus, and at some point during the evolution they had move into the organelles. But in the mid-1970s it was showed that this was not so. The scientists could prove that the genes inside the mitochondria and chloroplasts have little likeness to the genes in the nucleus. These findings were the base for one of the most accepted theories about the mitochondrial origin - the theory of endosymbiosis. According to this theory, an endosymbiont - a primitive prokaryote invaded a host cell, thereby establishing a symbiotic relationship that is an origin of the eukaryotic cell. In this model, the endosymbiont DNA from chloroplast and mitochondria, is believed to be related to cyanobacterial and proteobacterial DNA respectively (fig 1).

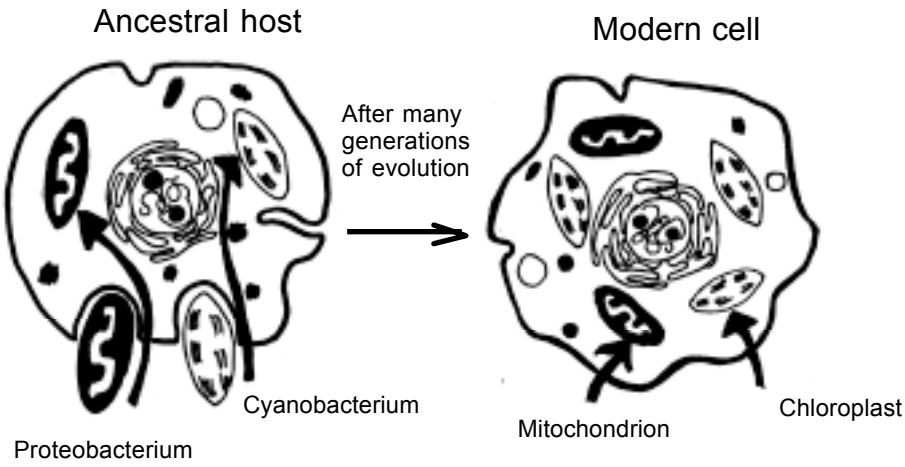

Figure 1. Endosymbiosis. Schematic picture of the endosymbiosis theory. Redrawn by Madeleine Johansson from (Margulis, URL)

Siv Andersson *et al*. (1998) sequenced the closest relative of mitochondria yet known: *Rickettsia prowazekii*, a bacterium that causes typhus. Some of the mitochondrial genes produce enzymes that build new mitochondrial DNA and RNA. However, those DNA and RNA building genes are not similar to the genes of related bacteria. They are virus-like genes (Filee & Forterre, 2005). This finding opened a new feature for the mitochondrial origin. The question of whether the mitochondria originated from a primitive bacterial event, or from a simultaneous event where also viruses were incorporated has thereby raced.

Table 1. *The history of mitochondrial physiology*

1774	Joseph Priesly and Antoine Lavoisier, discover oxygen and respiration
1857	Rudolph Kölliker, pioneer of light microscope, finds mitochondria in muscle
1890	Richard Altmann, develops mitochondrial stain, postulates genetic autonomy
1898	Carl Benda named mit. from Greek *mitos* "thread" and *khondrion* "little granule"
1924	Crude mitochondrial isolation (Warburg *et al.*, 1924)
1943	Isolation of intact liver mitochondria and microsomes (Claude, 1943)
1949	Localization of ß-oxidation, TCA and oxphos (Kennedy & Lehninger, 1949)
1952	Inner, outer membranes and cristae defined by EM (Palade, 1952)
1955	First mitochondrial NDPK activity reported (Herbert *et al.*, 1955)
1981	Endosymbiotic theory of mitochondrial origins (Margulis, 1981)
1981	Human mtDNA sequenced (Anderson *et al.*, 1981)
1986	First mtDNA diseases reported (Ikeda *et al.*, 1986)
1997	*Arabidopsis thaliana* genome sequenced *(Unseld et al., 1997)*
2000	*Beta vulgaris* genome sequenced *(Kubo et al., 2000)*
2002	*Oryza sativa* genome sequenced *(Notsu et al., 2002)*
2003	*Brasica napus* genome sequenced *(Handa, 2003)*
2004	*Zea mays* genome sequenced *(Clifton et al., 2004)*

Mitochondrial structure

The mitochondrion has two membranes dividing the organelle into a narrow intermembrane space (IMS) and much larger internal matrix (fig 2) each of which contains highly specialized proteins. The outer membrane contains many channels which makes the membrane permeable to molecules smaller than 10 kDa. The inner membrane contains protein complexes responsible for respiration, ATP production and transport of substrates into the matrix. The inner membrane forms a large number of infoldings called cristae.

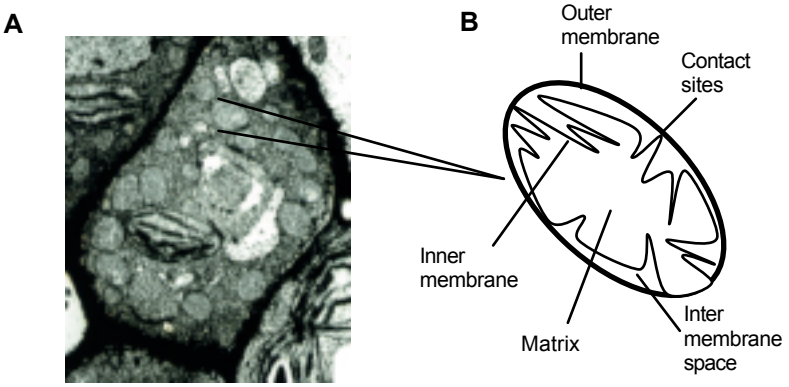

Figure 2. Mitochondrial structure. A) Plant cell, leaf tissue from spinach (*Spinacia oleracea)*. B) Mitochondrial compartments.

In mitochondria there are contact points between the outer and inner membranes. The contact sites were first described in the late 1960s (Hackenbrock, 1968). To date, the knowledge about the exact function and composition of these points is still poor. They are multi-protein complexes required for specific mitochondrial functions such as transport of proteins, solutes and energy. (Brdiczka *et al.*, 1998; Voisine *et al.*, 1999). Four different types of contacts have been described: (1) morphological contacts visualized by electron microscopy. These include unknown stable complexes (Hackenbrock, 1968). (2) Contacts that are involved in the translocation of proteins, including the translocases of the outer membrane and the translocases of the inner membrane (Rapaport, 2002). (3) Contacts coordinating fusion and fission events of mitochondria, where very little is known about the protein complexes (4) and contacts for channelling metabolites from the matrix to the cytoplasm and *vice versa*. The latter contain multi-protein channels, the permeability transition pores, which include porin, ATP/ADP carrier, and kinases such as hexokinase and creatine kinase (Adams *et al.*, 1989).

Genome

Mitochondrial genomes vary in size between organisms where the plant genomes are much bigger (180-2400 kb) than the animal ones (15-16 kb) (Wolstenholme & Fauron, 1995). Although mitochondria have their own genome, most of the mitochondrial proteins are nuclear encoded. After translation in the cytosol these proteins are imported into the organelle. One of these protein is the Nucleoside diphosphate kinase (NDPK).

Nucleoside Diphosphate Kinase

In the 1950s an enzyme activity was observed that transferred a phosphate onto another protein in a biological reaction called phosphorylation (Burnett & Kennedy, 1954). The protein responsible was a liver enzyme that catalyzed the phosphorylation of casein and became known as a protein kinase, the first of its kind to be observed.

Today it is known that the transfer of phosphates onto proteins is catalyzed by a variety of enzymes that share certain characteristics and fall into the class of protein kinases. Their similarities stem from the ability to take a phosphate from an energy-carrying molecule (ATP/GTP) and place it onto an amino acid side chain of a protein. The hydroxyl groups (-OH) of serine, threonine, histidine or tyrosine amino acid side chains are the most common targets (fig 3). A second class of enzymes is responsible for the reverse reaction where phosphates are removed from a protein. These are named protein phosphatases.

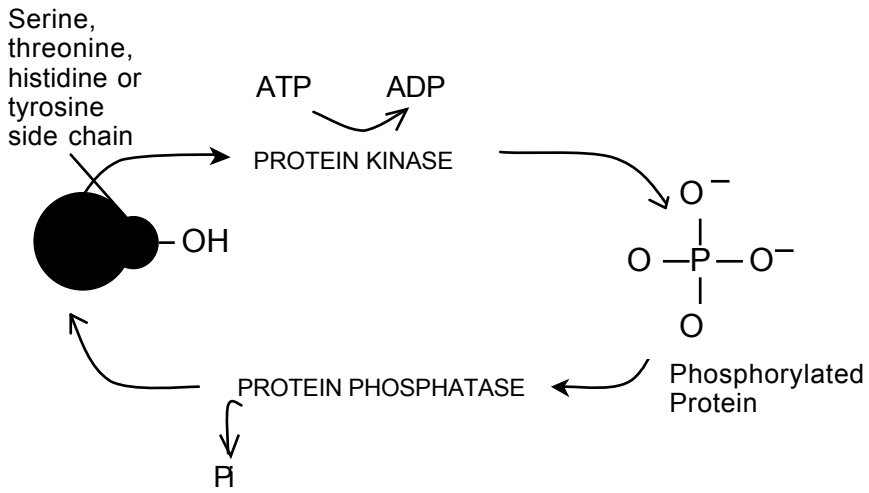

Figure 3. Reversible phosphorylation.

The kinase activity of NDPK

NDPKs are enzymes that in the presence of Mg^{2+} catalyze the transfer of phosphate groups between nucleoside phosphates. The reaction can be summarized as followed:

$N_1TP + E \leftrightarrow N_1DP + E\text{-}P$
$N_2DP + E\text{-}P \leftrightarrow N_2TP + E$ (N = G, A, T, C and U)

The active sites of NDPK bind the γ-phosphate from nucleoside triphosphate. The nucleoside triphosphate, now diphosphate, is released, and a different nucleoside diphosphate binds to the same site. As result the phosphate that is bound to the enzyme is transferred to the new diphosphate, forming a new triphosphate. This catalytic reaction is called a ping-pong mechanism (Parks & Agarwal, 1973).

Phosphorylation of NDPK

Each NDPK monomer has a single nucleoside triphosphate binding site important for the phosphorylation of the enzyme (Parks & Agarwal, 1973). Phosphorylation of NDPK on its active histidine residue has been widely accepted as part of its catalytic mechanism (Morera *et al.*, 1995). However, serine phosphorylation was also reported in NDPKs from various species including human NDPK nm23-H1 (MacDonald *et al.*, 1993) and *Myxococcus xanthus* NDPK (Munoz-Dorado *et al.*, 1993). In plants, serine and threonine phosphorylation of NDPK residues have been reported in *B. vulgaris* NDPK (Moisyadi *et al.*, 1994), *S. oleracea* NDPK2 from chloroplast (Bovet & Siegenthaler, 1997) and *P. sativum* NDPK3 from mitochondria (Struglics & Håkansson, 1999). The Ser119 and Ser69 are completely conserved in human nm23-H1-H6 but also in the *P. sativum* sequence. The residue corresponding to Ser44 in human nm23-H1, which is the major serine phosphorylation site (MacDonald *et al.*, 1993) is not conserved and is in the *P. sativum* sequence replaced by threonine. It has been suggested that the phosphorylation of Ser44 is involved in the suppression of tumour metastasis (MacDonald *et al.*, 1993). The crystal structure of nm23-H2 shows that Ser44 is located on the top of a groove containing the catalytic His118 (Webb *et al.*, 1995). Since the putative serine phosphorylation site is located closely to the active site histidine residue in the nucleotide binding pocket of NDPK, a phosphotransfer to the serine residue cannot be excluded (Shen *et al.*, 2006). It is possible that the serine phosphorylation results in conformational changes of NDPK, resulting in opening of the groove to make phosphorylation of the conserved histidine possible. In paper II we describe the residues involved in serine phosphorylation of NDPK3 from *P. sativum*.

NDPK as a multifunctional enzyme

NDPKs are found in different species of vertebrates, bacteria and plants but have been mostly studied in mammals. In humans there are eight different isoforms (table 2) and in *A. thaliana*, there are five (table 3). NDPKs exhibit various regulatory functions that may be related to, or independent of, their catalytic activity (Agou *et al.*, 1999; Postel *et al.*, 2002; Postel *et al.*, 2000). Many of these functions are mediated by protein-protein interactions between NDPKs and other proteins. These interactions can modulate the activity of other proteins such as the chaperon function of hsp70 (Leung & Hightower, 1997). In humans, NDPK was identified as a tumour metastasis suppressor (Steeg et al., 1988), transcriptional activator of *c-myc* (Agou *et al.*, 1999; Postel *et al.*, 1993) and supplier of GTP to G-proteins. Furthermore, both mammalian and bacterial NDPK can bind and cleave DNA (Levit *et al.*, 2002; Postel *et al.*, 2002)

The plant NDPKs are also multifunctional enzymes involved in hormone signalling (Nato *et al.*, 1997; Novikova *et al.*, 2003), UV-light light response (Zimmermann *et al.*, 1999) and interaction with phytochromes (Choi *et al.*, 1999; Shen *et al.*, 2005). The pea mitochondrial NDPK3 is involved in response to heat stress via the interaction with an 86 kDa protein (Escobar Galvis *et al.*, 2001) and is able to bind cAMP (Knorpp & Håkansson, 1998; Laukens *et al.*, 2001).

Table 2. *The human NDPK isoforms. The accession numbers for human NDPKs are: P15531 (nm23-H1), P22392 (nm23-H2), Q13232 (nm23-H3), O00746 (nm23-H4), P56597 (nm23-H5), O75414(nm23-H6), Q9Y5B8 (nm23-H7), O60361 (nm23-H8).* [1]*Nd, not determined*

Isoform	**Sub-cellular localization**	**Disease relevance / Function**	**References**
nm23-H1	Cytosol	Tumour progression and metastasis	(Venturelli *et al.*, 1995)
nm23-H2	Cytosol Nuclear	Tumour progression and metastasis, gene regulation and apoptosis	(Venturelli *et al.*, 1995) (Lacombe *et al.*, 2000)
nm23-H3	Cytosol	Chronic myelogenous leukaemia	(Lacombe *et al.*, 2000) (Venturelli *et al.*, 1995)
nm23-H4	Mitochondrial	Gastric and colon cancer	(Seifert *et al.*, 2005)
nm23-H5	Nd[1]	Involved in early stages of spermatogenesis	(Munier *et al.*, 1998)
nm23-H6	Cytosol Mitochondrial	Gastric and colon cancer	(Seifert *et al.*, 2005) (Lacombe *et al.*, 2000)
nm23-H7	Nd[1]	Gastric and colon cancer	(Seifert *et al.*, 2005)
nm23-H8	Nd[1]	Nd[1]	(Lacombe *et al.*, 2000)

Table 3. *The Arabidopsis thaliana NDPK isoforms. The accession numbers for A. thaliana NDPKs are: P39207 (NDPK1), O64903 (NDPK2), AJ012758 (NDPK1a), O49203 (NDPK3a), Q8LAH8 (NDPK3b).* [1]*Nd, not determined*

Isoform	Sub-cellular localization	Function	References
NDPK1	Cytosol	Component of ROS signalling	(Fukamatsu et al., 2003)
NDPK2	Chloroplastic	Phytochrome response	(Choi et al., 1999)
NDPK1a	Cytosol Nuclear	H_2O_2 mediated MAPK signalling UV-light signalling	(Moon *et al.*, 2003) (Zimmermann *et al.*, 1999)
NDPK3a	Mitochondrial Chloroplastic	Heat shock response	(Escobar Galvis *et al.*, 2001) (Spetea *et al.*, 2004) (Paper III)
NDPK3b	Mitochondrial Putative chloroplastic	Nd[1]	(Hasunuma *et al.*, 2003) (Paper III)

Sub-cellular localization of NDPK

In eukaryotic cells NDPKs are located in the cytosol (Troll *et al.*, 1993) microsomes, (Lambeth *et al.*, 1997) plasma membrane (Kimura, 1993), nucleus, (Nosaka *et al.*, 1998) chloroplasts (Yang & Lamppa, 1996) and mitochondria (Lambeth *et al.*, 1997).

Plants have been found to contain three groups of NDPKs (Escobar Galvis *et al.*, 1999). NDPK1 in the cytosol (Tanaka *et al.*, 1998; Zimmermann *et al.*, 1999), NDPK2 in the cytoplasm, nucleus and chloroplast stroma (Choi *et al.*, 1999; Yang & Lamppa, 1996) and NDPK3 in the lumen of the chloroplast and mitochondrial intermembrane space (Spetea *et al.*, 2004; Struglics & Håkansson, 1999; Sweetlove *et al.*, 2001; Yang & Lamppa, 1996). Most studies have concerned the cytosolic isoforms of NDPK but also the mitochondrial enzymes. NDPK3 in plant mitochondria exists both as a soluble form in the IMS and membrane-bound form attached to the inner membrane (Struglics & Håkansson, 1999), (Paper I).

In *A. thaliana*, a similar nomenclature (NDPK1 and NDPK1a) is used for two isoforms of NDPK (table 3). However, NDPK1 and NDPK1a show no more than 57% identity, whereas NDPK1a and NDPK2 differ in only two amino acids. The high similarity of NDPK1a to NDPK2 may reflect that these genes were cloned from two different ecotypes (*Landsberger erecta* versus *Columbia*). Nowadays, NDPK1a is obsolete and classified as an NDPK2 gene (Hasunuma *et al.*, 2003; Tair-homepage).

Structure of NDPK

Most of the NDPK genes code for small proteins (14 – 19 kDa). The sequences of NDPK genes from various organisms have revealed that most of these proteins consist of approximately 150 amino acids extensively conserved from bacteria to human. NDPKs share primary, secondary and tertiary structure but differ in the quaternary structure. Based on the crystal structures, NDPKs are tetramers in prokaryotes (Giartosio *et al.*, 1996) and hexamers in eukaryotes (Webb *et al.*, 1995). All have very similar three-dimensional structures, and their subunits retain a characteristic fold with α-helices packed on four antiparallel ß-sheets (Dumas *et al.*, 1992). Three dimers associate to generate the hexamer whereas two dimers associate to generate the tetramer. The residues associated with hexamer formation have been remarkably conserved during evolution. The main difference between the hexameric and tetrameric NDP kinases is the C-terminal part of the molecule. In the hexameric NDPKs, the amino acids at the C-terminus interact with the neighbouring dimer, contributing to hexamer stability. In tetramers, the corresponding C-terminal is shorter and interacts with the neighbouring subunit of the same dimer (Lascu *et al.*, 2000). The quaternary structure is important for the stability of the protein. In *Drosophila melanogaster*, NDPK is the product of the *awd (abnormal wing discs)* gene, which is essential for development as mutations lead to larval lethality (Dearolf *et al.*, 1988). A natural point mutation in *awd* at residue P67 is called *K-pn* (*Killer of prune*). The *K-pn* substitutes a serine for a proline on a surface loop, named for this reason the K-pn loop. The conserved proline is found at position 95 in the pea mitochondrial NDPK3 (Paper II; fig 4). The hexameric structure is necessary for full enzyme activity (Mesnildrey *et al.*, 1998). In mammals, the K-pn loops are in the area of contact between the subunits and play an important part in the stability of hexamers (Karlsson *et al.*, 1996; Lascu *et al.*, 1992). Also in plants the trimer interactions are mediated through interactions of helix A5 with the Killer of prune loop (Paper II). The proline residue is conserved in most NDP kinases, but it is a serine in nm23-H4 (Milon *et al.*, 2000; Milon *et al.*, 1997), (fig 4). Mutation of the serine to a proline considerably increased protein stability (Milon *et al.*, 2000). In paper II we present the first plant mitochondrial X-ray structure of plant mitochondrial NDPK3.

Targeting of NDPK3

NDPKs and most of the mitochondrial and chloroplast proteins are nuclear encoded. After translation in the cytoplasm, transport to the organelle is carried out by selective import mechanisms (Glaser *et al.*, 1998). The targeting signals are usually present in the N-terminal extensions of the protein. The mitochondrial targeting extension called pre-sequence, and chloroplastic named transit peptides, direct the respective protein to the given organelle (Bruce, 2001). When the precursor protein is transported to the organelle, its pre-sequence is removed by a peptidase, resulting in the mature protein form. It is believed that the secondary structure rather than the primary sequence of the pre-sequence determines targeting.

Because mitochondria and chloroplasts share some overlapping functions, such as DNA replication, transcription, translation, energy production and protection from oxidative stress (Akashi *et al.*, 1998), some enzymes are found in both organelles. There are two possible mechanisms by which dual targeting can be achieved: through either twin targeting sequence or an ambiguous targeting sequence. Twin targeting signals may be the result of alternative transcription or

translation initiation, alternative splicing, or a posttranslational modification that results in the formation of two proteins with distinct targeting peptides (Peeters & Small, 2001). The precursors carrying an ambiguous targeting signal exist as a single polypeptide form but can be recognized and transported to more than one organelle (Akashi *et al.*, 1998).

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P. sativum    NDPK3       --AELERTFIAIKPDGVQRGLISEIISRFERKGFKLVGIKVLIPTKQFAQQHYHDLKER
A. thaliana   NDPK3a      --AEMERTFIAIKPDGVQRGLISEIISRFERKGFKLVGIKVIVPSKDFAQKHYHDLKER
A. thaliana   NDPK3b      --AEMERTFIAIKPDGVQRGLISEIITRFERKGYKLVGIKVMVPSKGFAQKHYHDLKER
H. sapiens     nm23-H2    -MANLERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVAMKFLRASEEHLKQHYIDLKDR
P. sativum    NDPK1       ---MAEQTFIMIKPDGVQRGLVGEIISRFEKKGFYLKGLKFVNVERAFAEKHYADLSAK
P. sativum    NDPK2       ---QVDQAYIMVKPDGVQRGLVGEIISRFEKKGFKLTGLKLFQCSKELAEEHYKHLNQK
H. sapiens     nm23-H4    ---TRERTLVAVKPDGVQRRLVGDVIQRFERRGFTLVGMKMLQAPESVLAEHYQDLRRK
E. coli        NDPK        ---AIERTFSIIKPNAVAKNVIGNIFARFEAAGFKIVGTKMLHLTVEQARGFYAEHDGK

P. sativum    NDPK3       PFFNGLCDFLSSGPVIAMVWEGEGVITYGRKLIGATDPQKSAPGTIRGDLAVVVGRNI
A. thaliana   NDPK3a      PFFNGLCDFLSSGPVIAMVWEGDGVIRYGRKLIGATDPQKSEPGTIRGDLAVTVGRNI
A. thaliana   NDPK3b      PFFNGLCNFLSSGPVVAMVWEGEGVIRYGRKLIGATDPQKSEPGTIRGDLAVVVGRNI
H. sapiens     nm23-H2    PFFPGLVKYMNSGPVVAMVWEGLNVVKTGRVMLGETNPADSKPGTIRGDFCIQVGRNI
P. sativum    NDPK1       PFFSGLVDYIISGPVVAMIWEGKNVVTTGRKIIGATNPAQSEPGTIRGDFAIDIGRNV
P. sativum    NDPK2       SFFPKLIEYITSGPVVSMAWEGVGVVPSARKLIGATDPLQAEPGTIRGDFAVQTGRNI
H. sapiens     nm23-H4    PFFPALIRYMSSGPVVAMVWEGYNVVRASRAMIGHTDSAEAAPGTIRGDFSVHISRNV
E. coli        NDPK        PFFDGLVEFMTSGPIVVSVLEGENAVQRHRDLLGATNPANALAGTLRADYADSLTENG

P. sativum    NDPK3       IHGSDGPETAKDEIKLWFKPEELVSFTSNSEKWIYGDN
A. thaliana   NDPK3a      IHGSDGPETAKDEISLWFKPQELVSYTSNSEKWLYGDN
A. thaliana   NDPK3b      IHGSDGPETAKDEISLWFKPEELVSYTSNAEKWIYGQN
H. sapiens     nm23-H2    IHGSDSVKSAEKEISLWFKPEELVDYKSCAHDWVYE--
P. sativum    NDPK1       IHGSDAVESANKEIALWF-PEGAANWESSLHSWIYE--
P. sativum    NDPK2       IHGSDSPENGEREIALWFKEGELCEWTPVQEPWLRE--
H. sapiens     nm23-H4    IHGSDSVEGAQREIQLWFQSSELVSWADGGQHSSIHPA
E. coli        NDPK        THGSDSVESAAREIAYFFGEGEVCPRTR----------
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Figure 4. Sequence alignment of eight NDPK isoforms. The conserved amino acids Ser69, Ser119, and the active His117 are highlighted in red and the conserved Killer of prune Pro is bold. The sequences are trimmed and numbered according to the mature pea mitochondrial NDPK3 sequence, starting with the Alanine. Accession codes for the sequences are: AAF08537, mitochondrial pea NDPK3; O49203, mitochondrial Arabidopsis NDPK3a; Q8LAH8, mitochondrial Arabidopsis NDPK3b; P22392, cytosolic human nm23-H2; CAA50511.1, cytosolic pea NDPK1; P47923, chloroplastic pea NDPK2; O00746, mitochondrial human nm23-H4; P0A763, *Escherichia coli* NDPK.

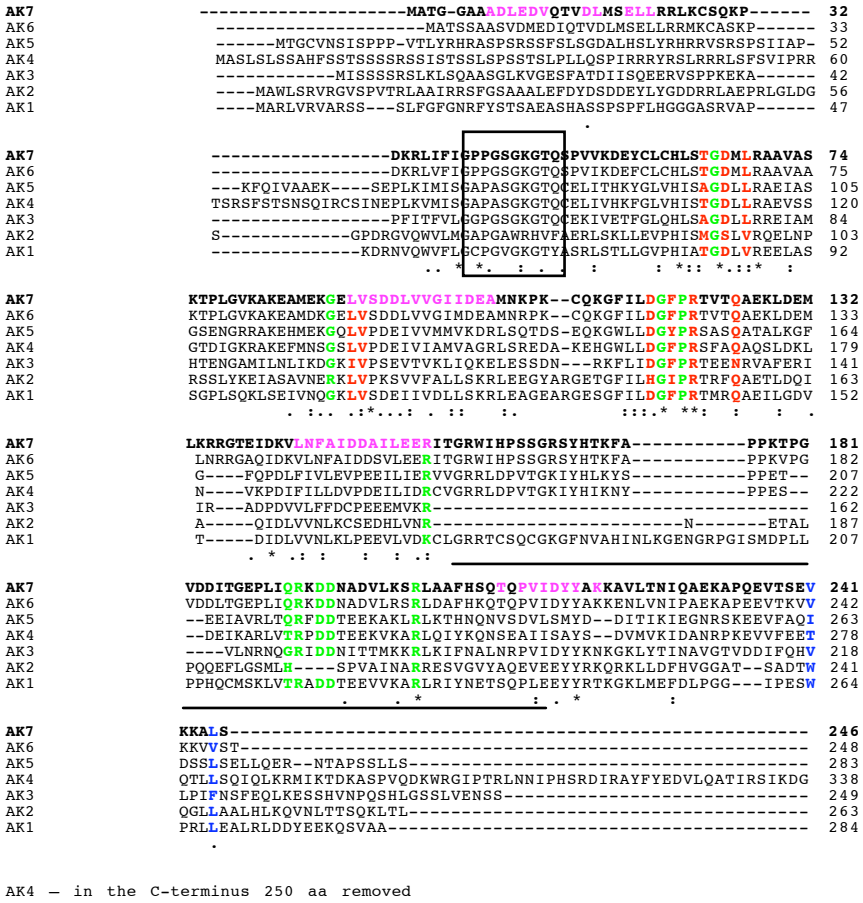
ATP/ADP translocator and Adenylate Kinase

The ATP/ADP translocator, also named the ATP/ADP carrier or adenine nucleotide translocator (ANT) is a member of the mitochondrial carrier family that transports ATP/ADP over inner mitochondrial membrane. The ATP/ADP translocator catalyses the exchange of cytosolic ADP for ATP synthesised in the matrix and thereby supports the cell with energy (Pfaff & Klingenberg, 1968).

Adenylate Kinase catalyzes reversible phosphotransfer between adenine nucleotides: ATP + AMP ↔ 2 ADP (Noda, 1973) The enzyme is an important component of the energy charge concept and maintains equilibrium of adenine nucleotides in the cell (Atkinson, 1968). At present, six isoforms with different subcellular localization have been described in mammalian tissues: AK1 and AK5 in the cytosol (Collavin *et al.*, 1999; Van Rompay *et al.*, 1999), AK2 in the mitochondrial IMS (Kohler *et al.*, 1999), AK3 and AK4 exclusively in the mitochondrial matrix (Noma *et al.*, 2001) and AK6 in nucleus (Ren *et al.*, 2005).

In plants, Adenylate Kinase has been identified in the stroma of chloroplasts (Hampp *et al.*, 1982), as well as in the cytosol and mitochondria (Stitt *et al.*, 1982). There are seven predicted Adenylate Kinases in *A. thaliana* genome named AK1 –7 (fig 5). Three of those, AK2, AK6 and AK7 are predicted to be located in

the mitochondria (Tair-homepage). AK4 that is predicted to have a chloroplast location is 250 amino acids longer than the other isoforms in *A. thaliana* (fig 5). Sequences producing significant alignments with these 250 amino acids were ATP binding nucleotide kinase from *A. thaliana* and a putative Adenylate Kinase, from rice chloroplast. However, no similarities with any of the *A. thaliana* Adenylate Kinase isoforms were found.



AK4 – in the C-terminus 250 aa removed

Figure 5. Sequence alignment of seven Adenylate Kinases from *A. thaliana*. The asterisks and dots represent residues that are identical or conserved in a majority of the sequences, respectively. The range of the binding and LID domains are defined according to the alignment by Ginger et al. (Ginger et al., 2005). The highly conserved ATP-binding loop is boxed, and the length of the LID domain is underlined. Other residues known to be important either in substrate binding or catalysis are highlighted by red and green, respectively. The blue shading reveals two conserved hydrophobic residues that form part of the essential C-terminal alpha helix, which makes hydrophobic contact with the purine ring of ATP. AK7, (bold), is the mitochondrial isoform identified as an interacting partner of the soluble NDPK3 (Paper IV). Similarities in amino acids between the peptides from *A. thaliana* and *P. sativum* (pink, bold), from MS-sequencing are indicated. The accession numbers for *A. thaliana* Adenylate Kinases were: Q9ZUU1 (AK1), 21928121 (AK2), 4454016 (AK3), 17979434 (AK4), Q9FIJ7 (AK5), Q9FK35 (AK6), O82514 (AK7).

Aims of the study

The general aim of the project was to study the functions and interacting partners of mitochondrial localized NDPK3, as one of the components of intracellular signalling in plants. Specific aims were to:

- Study whether NDPK3 in mitochondria of *P. sativum* exists in other sub-mitochondrial compartments than the IMS;
- Identify the residues involved in serine phosphorylation;
- Analyse the oligomeric state and structure of NDPK3;
- Provide mutational analyzes of enzymatically important conserved residues of NDPK3 and study the effects of the mutations on the enzyme activity and oligomerization;
- Characterize the evolutionary history, expression and targeting of NDPK3;
- Investigate the potential involvement of NDPK3 in contact sites of plant mitochondria;
- Identify interacting partners of NDPK3 in plant mitochondria and characterize the effects of the protein interaction.

Results and discussion

Sub-mitochondrial localization of NDPK3 in *P. sativum* (I)

Struglics and Håkansson (1999) purified the first plant mitochondrial NDPK3 isoform from pea and suggested localization to the IMS (Struglics & Håkansson, 1999). The IMS localization was later confirmed using a proteomics approach for mitochondrial NDPK from potato and *A. thaliana* by Sweetlove *et al*. (2001).

Sub-mitochondrial fractionation was used in order to investigate the presence of NDPK3 in other sub-mitochondrial compartments. The mitochondria were fractionated into membrane and soluble fractions by sonication and ultracentrifugation. Western blot with antibodies directed against the C-terminal of NDPK3 (Escobar Galvis *et al.*, 2001) was used determining the distribution. This experiment showed that in pea mitochondria there is as much NDPK3 in the membrane as in the soluble fraction (Paper I, fig 1A).

NDPK3 is firmly attached to the inner membrane of mitochondria

Mitoplasts were used in order to investigate the strength of the membrane association of NDPK3. Mitoplasts are mitochondria treated by osmotic shock so that the outer membrane ruptures, leaving the outside of the mitochondrial inner membrane exposed. The mitoplasts were washed with $NaCO_3$ and Triton X-100. Those chemicals generally remove the basic membrane proteins and hydrophobic proteins respectively. The results showed that the washes that abolished the membrane association of the peripheral inner membrane protein cytochrome *c* (Paper I, fig 2B) but only removed a small fraction of the membrane bound

protein (Paper I, fig 2A) inferring that NDPK3 is strongly attached to the inner membrane of mitochondria. This result was conformed by a proteinase K treatment of the mitoplasts. Also here just a minor fraction of NDPK3 was removed whereas the membrane association of cytochrome *c* was fully abolished (Hammargren, personal communication).

Due to the strong interaction of NDPK3 to the outer part of the inner membrane we were not able to investigate if the protein is also located in the matrix or not. It is also very difficult to isolate a pure soluble matrix fraction as this soluble fraction is easily contaminated by IMS. However, it cannot be excluded that NDPK3 is also attached to inner side of the inner membrane, facing the matrix.

Mutational analyses, serine phosphorylation, structure and oligomerization of NDPK3 (II, IV)

The signal transduction function of NDPK has been suggested to involve phosphorylation, not only on the active site histidine residue but also on a serine or threonine residue (MacDonald *et al.*, 1993). The aim of our study was to identify functionally important residues involved in the observed Ser phosphorylation (Struglics & Håkansson, 1999). We utilized the classic approach where EDTA, a chelator of divalent cations, was used in the phosphorylation assay (Francis *et al.*, 1989). Divalent cations such as Mg^{2+} are necessary for the NDPK activity and in their absence only autophosphorylation can occur as the phosphotransfer will stop on the enzyme residue(s) involved in the autophosphorylation (for more details see page 11; *The kinase activity of NDPK*). His117 (red in fig 4) was selected for site-directed mutagenesis in order to examine the phosphorylation of the conserved residues Ser69 and Ser119 and the active site histidine. Ser69 and Ser119 were replaced by Ala in order to maintain size without the potential to be phosphorylated. The active site His was mutated to Asp in order to maintain a similar size but inhibit all activity.

Enzymatic activity is a prerequisite for Ser phosphorylation

The recombinant purified mitochondrial NDPK3 proteins were assayed for alkali-stable His autophosphorylation and acid stable Ser autophosphorylation after incubation with [γ^{32}P]ATP in the presence of EDTA. The Ser autophosphorylation in the S119A mutant was 44% of the Ser autophosphorylation in wild type. This indicates that S119 is responsible for approximately one half of the phosphorylation but is not the only Ser to be phosphorylated. S119 is close to the nucleotide binding cleft and lies within 5Å of the active site H117. Thus the autophosphorylation of this residue is most likely a direct transfer via the phosphohistidine intermediate (Williams *et al.*, 1993). The level of S119A and S69A His phosphorylation was 120% and 6% respectively of the wild type. This was in agreement with the catalytic activities where S119A does not show a large change in specific activity compared to wild type whereas the S69A mutation resulted in a dramatic loss of enzymatic activity. This observation is in contrast to the human isoform nm23-H2, where the mutation of the corresponding Ser residue, Ser70, was found not to affect the catalytic properties of the protein (Postel *et al.*, 2002). No His or Ser autophosphorylation was detected for the enzymatically inactive H117D mutant. These results indicate that enzymatic activity is a prerequisite for autophosphorylation of serine.

The first crystal structure of plant NDPK3 from P. sativum

The first and so far only crystal structure of plant mitochondrial NDPK3 (Paper II) showed a structure similar to those of previously reported NDPKs (Lascu *et al.*, 2000). The crystal diffracts X-rays to 2.8 Å resolution where the asymmetric unit contain one hexamer. The six monomers of NDPK3 from *P. sativum* are arranged as trimers of dimers or dimers of trimers (Paper II, fig 1). The structure consists of a central core of a four stranded antiparallel ß-sheet surrounded by six α helices. The dimers interact through ß-sheet 2 and through the hydrogen bonding between helix A2. The trimer interactions are mediated through interactions of helix A5 with the Killer of prune loop (bold in fig 4) which is strictly conserved in most NDPKs (Lascu *et al.*, 1992).

S69 is important for the enzymatic activity and protein-protein interaction

It is known that there are correlations between function and structure of the NDPKs in mammals (Mesnildrey *et al.*, 1997). Escobar Galvis *et al*. (2001) showed that after gel filtration and immunodetection, NDPK3 was found in complexes of number of various sizes. We have observed similar results using size exclusion chromatography of the recombinant protein (not shown). The detected sizes of hexamers, tetramers and dimers indicate flexibility in oligomerization. The balance between the complexes is changed in the S69A where the mutation destabilizes NDPK3. In this mutant the dimer and tetramer is increased at the expense of the hexamer with a reduction of the enzymatic activity as a consequence.

In the pea mitochondrial NDPK3, similar to the human nm23-H2 structure, the S69 residue is exposed on the surface of the hexamer (Webb *et al.*, 1995). The S69 in the NDPK3 has only non-polar contact with Trp148 located in a bordering monomer and to the Phe66 in the same monomer. This interaction may by stabilizing the hexameric state of the enzyme. The destabilization of the hexameric structure with a more flexible loop caused by the Ser to Ala mutation may lead to changed ability of NDPK3 to bind substrate.

Adenylate Kinase was identified as an interacting partner of the soluble NDPK3 from the inter membrane space of mitochondria (Paper IV). The interaction modulates the activity of the enzymes where NDPK3 inhibits Adenylate Kinase. However the activity of Adenylate Kinase is unchanged when it interacts with S69A NDPK. This may indicate that the mutation affected the ability of NDPK:Adenylate Kinase interaction. Thus, the oligomeric state of NDPK3 is not just important for the activity of NDPK3 but also for the interaction with Adenylate Kinase.

Evolutionary history, expression and targeting of the plant NDPK family (III)

The plant NDPK gene family consists of three groups whose gene products end up in different sub-cellular locations. In *A. thaliana* cv. Colombia 0 there are four different NDPK isoforms named NDPK1-3 (table 3) where NDPK3a and NDPK3b are very similar. These isoforms differ by only 12 amino acids in the mature part of the proteins, none of which are in the active site (fig 6).

```
3a              MSSQICRSASKAAKSLLSSAKNARFFSEGRAIGAAAAVSASGKIPLYASNFARSSGSGVA 60
3b              MSSQICRSASRAARSLLSSAKNARFFSEGRAIGAASVVHATGKVPQYASNFGKS-GSGFV 59

3a              SKSWITGLLALPAAAYMIQDQEVLAAEMERTFIAIKPDGVQRGLISEIISRFERKGFKLV 120
3b              SNSWITGLLALPAAAFMLQDQEALAAEMERTFIAIKPDGVQRGLISEIITRFERKGYKLV 119

3a              GIKVIVPSKDFAQKHYHDLKERPFFNGLCDFLSSGPVIAMVWEGDGVIRYGRKLIGATDP 180
3b              GIKVMVPSKGFAQKHYHDLKERPFFNGLCNFLSSGPVVAMVWEGEGVIRYGRKLIGATDP 179

3a              QKSEPGTIRGDLAVTVGRNIIHGSDGPETAKDEISLWFKPQELVSYTSNSEKWLYGDN 238
3b              QKSEPGTIRGDLAVVVGRNIIHGSDGPETAKDEISLWFKPEELVSYTSNAEKWIYGQN 237
```

Figure 6. Sequence alignment of NDPK3a and NDPK3b from *A. thaliana*. The mature part of the proteins is marked by an arrow. Accession codes for the sequences are: O49203, Arabidopsis NDPK3a; Q8LAH8, Arabidopsis NDPK3b.

Separate evolution of NDPK3 in plants?

An earlier study of plant and vertebrate NDPKs showed that the plant isoforms are evolutionary more closely related to the vertebrate cytosolic form than to the vertebrate mitochondrial or to *Drosophila* nm23 (Escobar Galvis *et al.*, 1999). The amino acid sequences from 23 different *NDPKs* including monocots, dicots and moss were used in order to investigate the phylogeny of the *NDPK* gene family in plants. The *NDPKs* grouped in three major clades, *NDPK1, NDPK2* and *NDPK3* respectively. This grouping indicates that the function of NDPKs in the different subcellular compartments is well conserved. According to the evolutionary analysis, the NDPK3 proteins may have a somewhat more specific and conserved function as compared to the other NDPKs as it diverged erlier from the common ancestor (Paper III, fig 1).

The expression of the NDPK family in A. thaliana

The cytosolic *NDPK1* genes have been cloned from a variety of plant species. In tomato, *NDPK1* is highly expressed in both leaf and stem tissue (Harris *et al.*, 1994). Rice *NDPK1* expression changes during seed germination and in the early stages of seedling growth (Yano *et al.*, 1995). *NDPK1* represents more than half of the total NDPK transcript pool in leaves, roots and inflorescence tissues (Paper III).

Previous studies have shown that the the mitochondrial NDPK3 protein from *P. sativum* is more abundant in reproductive and young tissues than in vegetative and mature tissues (Escobar Galvis *et al.*, 1999). The expression pattern of NDPK3 in buds of *P. sativum* (Escobar Galvis *et al.*, 2001) is in agreement with the gene expression of *NDPK3a/b* homologues in *A. thaliana* (Paper III, fig 2A, 2B). The

NDPK3b, however, showed much weaker but distinct signal in tapetal, ovary and petal tissue (Paper III, fig 2B). Probably, NDPK3b can function as a complement to NDPK3a in tissues with high mitochondrial activity.

NDPK3 is dually targeted to mitochondria and chloroplasts

Protein targeting is usually highly specific. Nevertheless, a certain number of proteins are recognized by both mitochondria and chloroplasts. In plants, mitochondrial targeting sequences are generally longer than in other organisms, 40 amino acids on average (Glaser *et al.*, 1998). The mitochondrial *P. sativum* pre-sequence is 80 amino acids, containing an intramitochondrial targeting part (Escobar Galvis *et al.*, 1999). The mitochondrial pre-sequence is commonly rich in argenine and poor in acidic amino acids and contain aliphatic residues (leucine and alanine). The structure of the pre-sequence is usually an amphiphilic helix (von Heijne *et al.*, 1989). The C-terminus of the *P. sativum* pre-sequence is predicted to form a helix-kink-helix motif (Paper III). A helix close to the processing site has in some cases been shown to be important for recognition by the mitochondrial processing peptides (MPP) (Tanudji *et al.*, 1999) where the argenine at the -2 or -8 position from the cleavage site is critical.

The chloroplast targeting sequences are generally about 50 amino acids long, rich in serine and poore in leucine residues and in contrast to mitochondria, they do not contain many positive charged residues in the first ten amino acids (von Heijne & Nishikawa, 1991). The way to obtain dual targeting is to have a targeting sequence that is recognized as an import signal by both mitochondria and chloroplasts.

As the NDPK3 protein has been found in both the lumen of the chloroplasts and in the IMS of mitochondria (Spetea *et al.*, 2004; Struglics & Håkansson, 1999; Sweetlove *et al.*, 2001; Yang & Lamppa, 1996), we wanted to study if the protein is dual targeted or not. A NDPK-GFP construct was transformed into *A. thaliana* protoplasts. As shown in Paper III (fig 3), the NDPK3 is able to direct the GFP to both mitochondria and chloroplasts. Western blot analyses of pea subcellular fractions confirmed the dual localization of NDPK3, using NDPK3 antibodies produced against the C-terminus of the protein. The enzyme was not detected in the stroma but in the chloroplast thylakoid fraction. Even so, the majority of the enzyme was detected in the mitochondrial fraction (Paper III, fig 4).

Interacting partners of mitochondrial NDPK3 from *P. sativum* (I, IV)

Many of the regulatory functions of NDPK are mediated by protein-protein interactions (Leung & Hightower, 1997). For the interaction studies it is important to know that the proteins are located in the same compartment. We showed that the NDPK3 in plant mitochondria exists both attached to the inner membrane, where it interacts with the ATP/ADP translocator (Paper I), and soluble in the IMS, where Adenylate Kinase is an interacting partner (Paper IV).

Interaction between NDPK3 and ATP/ADP translocator

Mitochondrial membrane fraction from *P. sativum* was used in a co-immunoprecipitation experiment in order to identify the membrane proteins that NDPK3 interacts with. The interacting protein was in gel digested followed by QTOF-MS analysis. Five peptide sequences from the protein were obtained. The sequences were most similar to *A. thaliana* ATP/ADP translocators with accession number P31167. The sequences covered 17% of the Arabidopsis protein and were found to be 87% identical. The antibodies directed against ATP/ADP translocators were used in order to confirm the interaction of this protein with NDPK by cross-immunoprecipitation (Paper I, fig 4). Based on these results we conclude that the ATP/ADP translocator is an interacting partner of the membrane bound NDPK3.

Mitochondrial NDPK3 - a part of the contact point complex?

It has been shown that fractions containing the metabolite transport contact sites include various proteins and marker enzymes such as porins, creatine kinases and ATP/ADP translocators (Uribe *et al.*, 2003). NDPK3 from pea mitochondria is strongly attached to the inner membrane (Paper I). This could indicate that NDPK3 together with the interaction partner, ATP/ADP translocator, are localized to the contact points for channelling metabolites from the matrix to the cytoplasm and *vice versa*.

We isolated four fractions of mitochondrial membranes by osmotic shock treatment followed by sucrose gradient ultracentrifugation (fig 7A). In order to examine whether NDPK3 co-localizes with the markers of the metabolite contact points or not, the fractions were investigated by western blots where specific antibodies against ATP/ADP translocator, porin, F1 and NDPK3 were used (fig 7B). The different fractions were further investigated by partial detergent solubilization and size exclusion chromatography. ATP/ADP translocator, porin, F1 and NDPK3 were found to overlap (fig 7C). This overlap may suggest that NDPK3 is related to the contact sites with porin and ATP/ADP translocator as the marker enzymes. This interaction may have effect on ATP concentration on the IMS side of the inner membrane and thereby facilitating a higher rate of ADP/ATP exchange. However, the specific link with the contact site components needs to be further isolated and analysed in order to define the specific role of NDPK3, the interaction with the ATP/ADP translocator (Paper I) and other interacting proteins.

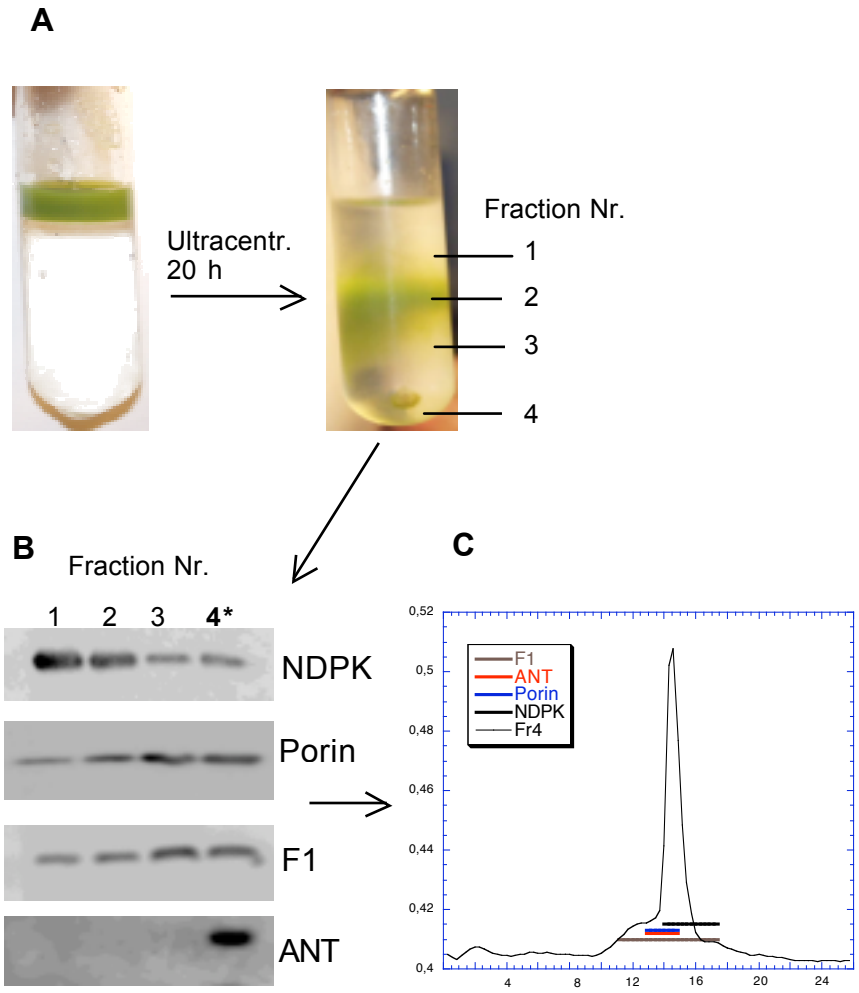


Figure 7. Fractionation of mitochondrial membranes from *P. sativum*. A) Separation of mitoplast fraction. Sonicated mitoplasts were separated by ultracentrifugation at 20h on 55:40% sucrose gradients. B) Western blots analyses. Four different fractions were analyzed by western blot with antibodies directed against NDPK3, Porin, F1 and ANT. Equal protein amount was loaded. In fraction four, marked by asterix, all the enzymes were detected. C) FPLC analyses. Fraction four was partially solubilized by 1% Triton X-100 for 15 min and separated on a Superose 6 column in the presence of 0.1% Triton X-100. Fraction Nr 14 and 15 contains all four enzymes; F1, ANT, Porin and NDPK3.

Interaction between NDPK3 and Adenylate Kinase

In order to find putative mitochondrial interaction partners for soluble mitochondrial NDPK3 we used affinity chromatography. The mitochondrial IMS fraction was passed through an affinity column with covalently bound recombinant NDPK3. The interacting proteins were eluted by salt gradient and identified by MS-QTOF sequencing. The peptide sequence (fig 5) was most similar to *A. thaliana* Adenylate Kinase with a sequence similarity of 90% between the *P. sativum* peptide sequences and *A. thaliana* gene At5g63400.

Structure of Adenylate Kinase

Several Adenylate Kinase isoforms have been crystallised as monomers with an exception for one reported trimer in Sulfolobus bacteria (Bonisch *et al.*, 1996). The monomer is composed of three sub-domains (Schulz *et al.*, 1990): the AMP binding domain, the lid domain and the core domain that is unaffected by substrate binding (Vonrhein *et al.*, 1995). The active site is located at the end of a channel, deep in the structure which closes around AMP and ATP, shielding the reaction from water (Bergman, 1999). The movement of the AMP binding site LID domain induced by substrates leads to two conformations called *closed* and *open* states (Schulz, 1992). There are size variations among the Adenylate Kinases called long and short isoforms. The long and short types of Adenylate Kinases differ in the LID domain. LID is an eleven residue segment in short type, whereas it is longer in the long type (Fukami-Kobayashi *et al.*, 1996). The LID domain is predicted to be 80 amino acids in *A. thaliana* family (fig 5), which indicates that these isoforms belong to the long type Adenylate Kinases. In plants, there is only one structure of Adenylate Kinase available (Wild *et al.*, 1997). This isoform is a monomer. In order to investigate the oligomeric state of Adenylate Kinase from *A. thaliana*, chemical cross-linking with glutaraldehyde (GA) was performed. Surprisingly, the recombinant His-tagged enzyme gave four distinct bands, showing a cross-linking pattern corresponding to a tetrameric structure (fig 8). It would be the first known tetramer not just in plants but also in the Adenylate Kinase family. However, this result needs to be further analyzed by *e.g.* size exclusion chromatography or native gels in order to confirm the unusual oligomeric state of the enzyme.

NDPK3 and Adenylate Kinase as cooperating partners

In paper IV we show that the interaction of NDPK3 and Adenylate Kinase modulates the activity of the enzymes and their responses to cAMP and calcium. In mammalian systems there is cooperation between NDPK and Adenylate Kinase activities in the mitochondria needed for facilitation of *e.g.* protein import into the nucleus (Dzeja & Terzic, 2003). A direct interaction between the viral Adenylate Kinase and *E. coli* NDPK was recently shown after T4 infection. Both enzymes were a part of T4 dNTP synthetase, a multienzyme complex which facilitates the synthesis of dNTPs and their flow into DNA (Kim *et al.*, 2005). This may indicate that the interaction between NDPK3 and Adenylate Kinase is a general feature and not specific to plants.

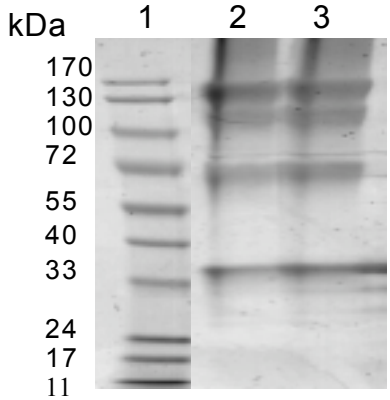


Figure 8. Cross-linking of Adenylate Kinase with glutaraldehyde (GA). GA amount in lanes 2 - 0.02% and 3 - 0.007%. Equal protein amount was loaded. The protein size marker, lane 1, and the relevant molecular mass (kDa) are indicated on the left.

Effects of cAMP and calcium on NDPK3 and NDPK:AK interaction

The coupled pyruvate kinase-lactate dehydrogenase assay (fig 9) was used in order to investigate the enzyme activity of NDPK3, Adenylate Kinase and the NDPK:Adenylate Kinase (NDPK:AK) interaction. The ADP production of the enzymes is indirect measured via the decrease of NADH where dTDP - the substrate for NDPK3 and/or AMP - the substrate for Adenylate Kinase are included.

The NDPK:AK interaction was measured in two different ways. First, in the absence of AMP or in the presence of the Adenylate Kinase specific inhibitor Ap5A, *e.g.* measuring the NDPK3 activity in the interacting complex. Second, in the absence of dTDP or in the present of enzymatically inactive NDPK3 mutant H117D (Paper II) *e.g.* measuring the Adenylate Kinase activity in the complex.

Second messengers such as cAMP and calcium are intracellular molecules which transmit signals in cells. cAMP is derived in a reaction where ATP can be broken down into non-toxic products as inorganic phosphate and AMP. The synthesis and degradation of cAMP is controlled by the enzymes named adenylate cyclase and

cyclic nucleotide phosphodiesterase, respectively (Abel *et al.*, 2000; Hanoune & Defer, 2001; Hetman *et al.*, 2000; Ichikawa *et al.*, 1997).

It has been shown that in tobacco two NDPK isoforms bind to cAMP where one of those is an orthologue of the NDPK from *P. sativum* (Laukens *et al.*, 2001). In *Dictyostelium,* the NDPK associated with the membrane was stimulated by cAMP, where the GTP produced by NDPK activated the G-proteins (Bominaar *et al.*, 1993). We observed that cAMP in the coupled pyruvate kinase-lactate dehydrogenase assay inhibited the activity of NDPK3 by itself and in the interaction with Adenylate Kinase (Paper IV; fig 1a, 4a).

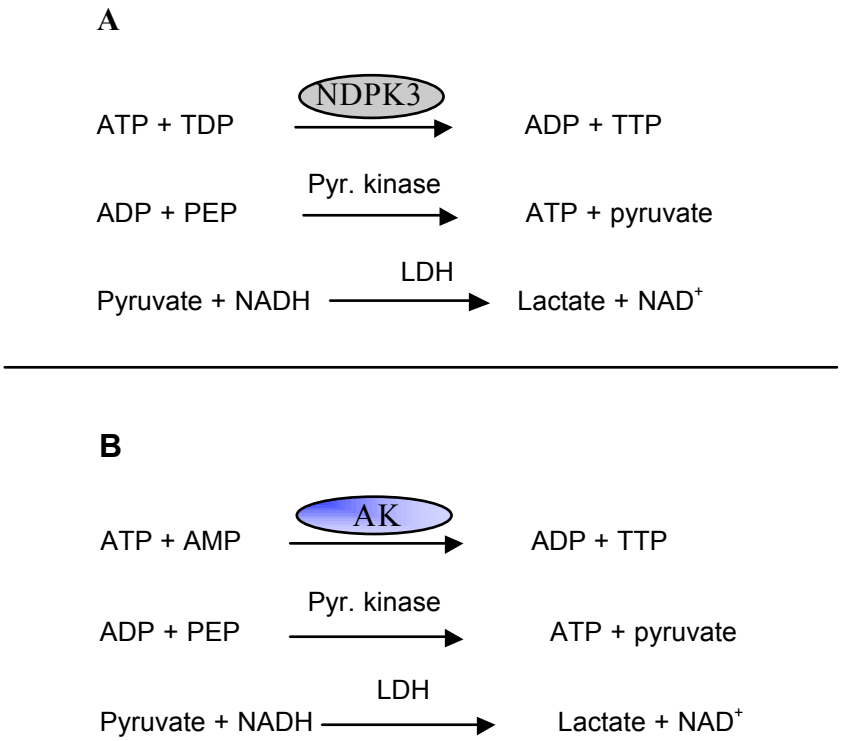


Figure 9. Summary of the coupled assay using pyruvate kinase and lactate dehydrogenase. ADP formed in the first step is phosphorylated to ATP by pyruvate kinase in the presence of phosphoenolpyruvate (PEP). Pyruvate is reduced in the presence of NADH by lactate dehydrogenase (LDH). A) Enzymatic assay for NDPK3. B) Enzymatic assay for Adenylate Kinase.

Calcium is a second messenger in many signalling pathways such as those activated by pathogen attack (Blume *et al.*, 2000), salt stress (Epstein, 1998), cold shock (Xiong *et al.*, 2002) and during pollen tube growth and root nodulation (Evans *et al.*, 2001). cAMP metabolism in higher plants is often connected to calcium levels where both the synthesis and degradation of the nucleotides are

controlled by concentrations of this ion (Kurosaki *et al.*, 1993). We observed that calcium inhibited NDPK3 activity itself and in the complex with Adenylate Kinase at concentrations under 0.5 μM. Higher concentrations of calcium showed no inhibitory effects (fig 1b, 4b). These inhibitions have direct effects on the GTP/ADP production in the IMS of mitochondria. As we show the inhibition of the enzymes and thereby the regulation of GTP/ADP amount occurs in two steps. First, low concentrations of the messenger results in low GTP/ADP production. Second, the enzyme activity and the GTP/ADP production is restored when higher concentrations are present (Paper IV, fig 1b, 3a, 4b). ADP formed in these reactions may be further consumed during oxidative phosphorylation with stimulation of respiration as a consequence (Jacobus & Evans, 1977). Adenylate Kinase stimulated the activity of NDPK3 and the activity of Adenylate Kinase was inhibited by NDPK3. However, the inhibition of Adenylate Kinase and the stimulation of NDPK3 in the interacting complexes occur at the same rate and thereby resulted in the same amount of ADP produced (Paper IV).

Conclusions

The main conclusions from the results presented in this thesis follow.

- The plant NDPK3 localizes to both the intermembrane space and to the mitochondrial inner membrane where it is firmly attached.
- The first crystal structure of a plant NDPK3 confirmed a hexameric oligomeric state of the enzyme.
- The Ser autophosphorylation depends on the enzyme activity.
- The mutation of the conserved Ser119 to Ala reduced the Ser phosphorylation to about one-half compared to wild type with only modest change of enzyme activity.
- Mutation of S69 to Ala reduced the enzymatic activity radically. This residue is also important for the oligomerization of NDPK3 and for the interaction of NDPK3 with Adenylate Kinase.
- *NDPK1, NDPK2* and *NDPK3* were present already in the last common ancestors of vascular plants and mosses.
- *NDPK3a* has the second highest expression in inflorescences, leaves and roots after *NDPK1*.
- *NDPK3b* expression is elevated in later stages of flower development in tapetum, ovules and petals.
- NDPK3 is dually targeted to mitochondria and chloroplasts where the major amount of the protein is found in the mitochondria.
- The enzymatic activity of NDPK3 is regulated by cAMP and calcium.
- The membrane associated NDPK3 interacts with ATP/ADP translocator.
- The IMS located NDPK3 interacts with Adenylate Kinase.
- The interaction of NDPK3 and Adenylate Kinase modulates the activity of the enzymes and their response to cAMP and calcium.

Future perspectives

Here we show increasing evidence that NDPK3 is an important multifunctional protein involved in cell signalling and coordination of other proteins. The study was performed to generate information about this mitochondrial isoform in plants, its biochemical characterisation and interacting partners. Additionally, the first structure of the enzyme provides possibilities for further studies of the protein folding. Furthermore, the discovered interacting proteins, ATP/ADP translocator and Adenylate Kinase may be an important link to more complex systems in the cell. I suggest the following research as examples of how to develop the material for further investigation.

Paper I

The resistance of NDPK3 to the washes could indicate localization to the contact points between the outer and inner mitochondrial membrane. It would be interesting to know if the ATP/ADP translocator that binds NDPK is the ATP/ADP translocator in the contact points or if it is an ATP/ADP translocator:NDPK complex by itself. The optimization of the assay with FPLC technique (fig 7) and use of different detergents could be utilized in order to separate the proteins in the contact points.

Paper II

The S69A mutation affects the oligomerization state of NDPK3. Increased flexibility of the head region could alter the trimer/hexamer interactions, thus forcing the equilibrium toward a dimer/tetramer structure. The mutation affected the enzymatic activity of NDPK3 and the interaction with Adenylate Kinase. Structural studies of the S69A mutant would confirm and clarify the oligomeric changes affecting the activity and the ability to interact with Adenylate Kinase.

Paper III

The translocation system of the NDPK3 over the chloroplast and mitochondrial membrane is most likely different. The C-terminal of the presequence contains a thylakoid processing peptidase like motif. The pea mitochondrial NDPK3 pre-sequence lacks the MPP cleavage residues, an argenine at the -2 or -8 position from the cleavage site. Escobar Galvis *et al*. (1999) suggested that the pea mitochondrial NDPK3 isoform might be processed by an inner membrane protease (IMP), in analogy to the one described by Nunnari *et al* (1993). The specificity of the IMP is such that it cleaves at -3 and -1 residue where an alanine at position -1 from the first amino acid of the mature protein is essential (Thompson *et al.*, 1999). Since the mitochondrial pea NDPK3 contains such an alanine, this pre-sequence may be a good candidate substrate for a putative plant mitochondrial signal peptidase. Processing studies where the cleavage residues would be mutated would clarify this subject.

Paper IV

The protein interaction of NDPK3 and Adenylate Kinase is an important link to the cross-talk between these kinases, cAMP and calcium. This interaction is probably a part of a multi-enzyme complex. It would be interesting to know if there are other members in the system or if it is just the AK:NDPK complex by itself. It would be important to start immunochemistry studies with antibodies directed against Adenylate Kinase. One other option would be to bind the Adenylate Kinase covalently to the NHS activated HP column in order to find the interacting partners from mitochondrial IMS. Putative interacting enzymes could be enzymes of the multi complex including Adenylate Kinase and NDPK.

Knowledge of the oligomeric state of Adenylate Kinase and the nature of NDPK:AK interaction is of crucial relevance to the potential study of the protein complex. The crystallization of the enzyme(s) would lead to the identification of residues important for the protein interaction. Size exclusion chromatography or blue native gels could confirm the unusual oligomeric state of Adenylate Kinase (fig 8).

It would also be interesting to know more details about Adenylate Kinase isoform AK4 (fig 5) as this predicted chloroplastic enzyme is 250 amino acids longer than the six other members of Adenylate Kinases in the *A. thaliana* family. It would be interesting to investigate the enzyme structure and the function of the C-terminal part, which has no similarities with *A. thaliana* Adenylate Kinase isoforms.

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