

# SIGNALLING IN PLANT MITOCHONDRIA

Redox regulation of gene expression & characterisation of a  
pea nucleoside diphosphate kinase

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## ABBREVIATIONS

A	adenine
aa	amino acid
AA	antimycin A
ADP	adenosine diphosphate
AltOx	alternative oxidase
ATP	adenosine triphosphate
cyt c	cytochrome c
C	cytosine
DBMIB	2,5-dibromo-3-methyl-6-isopropyl- <i>p</i> -benzoquinone
DC	dicumarol
DNA	deoxyribonucleic acid
E	glutamic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis-( $\beta$ -aminoethyl ether)
extDH	external NAD(P)H dehydrogenases
FAD	flavin adenine dinucleotide
FADH <sub>2</sub>	reduced flavin adenine dinucleotide
G	guanine
Hsp22	22 kDa heat shock protein
IMP	inner membrane protease
intDH	internal NAD(P)H dehydrogenases
kb	kilobase
kDa	kilodalton
lmw-hsp	low molecular weight heat shock protein
MPP	mitochondrial processing peptidase
mRNA	messenger RNA
mt	mitochondrial
mtHsp70	mitochondrial 70 kDa heat shock protein
mtNDPK	mitochondrial nucleoside diphosphate kinase
NAD(P)H	reduced nicotinamide adenine dinucleotise (phosphate)
NAD(P) <sup>+</sup>	oxidised nicotinamide adenine dinucleotise (phosphate)
NDP	nucleoside diphosphate
NDPK	nucleoside diphosphate kinase
nt	nucleotide
NTP	nucleoside triphosphate

OOA	oxaloacetate
ORF	open reading frame
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase
Pi	inorganic phosphate
PSI	photosystem I
PSII	photosystem II
RNA	ribonucleic acid
rRNA(s)	ribosomal RNA(s)
SHAM	salicylhydroxamic acid
T	thymine
TCA	tricarboxylic acid
TIM	translocase of the inner mitochondrial membrane
TOM	translocase of the outer mitochondrial membrane
tRNA(s)	transfer RNA(s)
TTFA	thenoyltrifluoroacetone
UQ (UQ-pool)	ubiquinone pool
U	uracil
UTP	uridine triphosphate
Y	tyrosine

## LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals:

- I Escobar Galvis ML, Allen JF & Håkansson G (1998) Protein synthesis by isolated pea mitochondria is dependent on the activity of respiratory complex II. *Curr Gen* 33: 320-329
- II Escobar Galvis ML, Allen JF & Håkansson G (1998) Mitochondrial gene expression is regulated by the activity of respiratory complex II. In: *Plant mitochondria: from gene to function*, IM Møller, P Gardeström, K Glimelius & E Glaser (eds) Backhuys publishers, Leiden, The Netherlands, pp. 171-174.
- III Escobar Galvis ML, Håkansson G, Alexciev K & Knorpp C (1999) Cloning and characterisation of a mitochondrial NDPK. *Biochimie* 81: 1089-1096.
- IV Escobar Galvis ML, Marttila S, Håkansson G, Forsberg J & Knorpp C (2000) Heat stress response in pea involves interaction of mitochondrial nucleoside diphosphate kinase (mtNDPK) with a novel 86 kDa protein. *Manuscript*.

## INTRODUCTION

Organelles are specialised compartments of the eukaryotic cell. Their outer membranes separate them from the cytosol. One of these organelles is the mitochondrion. It carries out respiration, providing energy to the cell. The mitochondrion is a semi-autonomous organelle that has its own genome and the machinery necessary for gene expression. It is believed that mitochondria arose from a bacterium that encountered the ancestor of the nucleo-cytoplasmic system of the eukaryotic cell. The encounter eventually resulted in the establishment of a symbiotic relationship (Gray, 1993). Even though, during evolution, most of the surviving bacterial genes have been transferred to the nucleus, some of the genes encoding components of the electron transport chain and the organellar gene expression machinery have been retained as the mitochondrial genome. In plant cells, mitochondria have evolved in the presence of a photosynthetic organelle, the chloroplast. Chloroplasts, like mitochondria, are semi-autonomous. Their origin is also believed to be endosymbiotic and a prokaryotic phototroph is considered as the chloroplast ancestor (Gray, 1993).

From the energetic point of view, the maintenance of the chloroplast and mitochondrial genomes is costly and must therefore confer some evolutionary advantage. A possible explanation for the retention of particular genes could be that their expression must be regulated *in situ*, in order to respond rapidly to changes in the environment which would be “sensed” by the organelles themselves (Allen, 1993b). The so-called “sensing” can be exerted by the organelles since the redox state of the components of their electron chains is directly affected by external factors (e.g. light, oxygen). The hypothesis that redox-regulated gene expression is the function of organellar genomes, predicts a regulatory system that enables the cell to sense environmental changes and to respond to those changes, by controlling the expression of organellar genes. This hypothesis has been suggested as an explanation for the presence of organellar genomes (Allen, 1993a).

Since most of the genetic information of the cell is contained and processed in the nucleus, co-ordination of nuclear and organellar gene expression is essential for the correct function of the cell. It has been

shown that the biogenesis and function of organelles are absolutely dependent on the nucleus (for a review see Leon *et al.*, 1998). The nucleus encodes most of the components that build up the organelles as well as components involved in regulation of organellar processes such as replication and gene expression. Thus, development of effective means of communication between the organelles and the nucleus must have been essential during evolution of the plant cell. A few examples of such communication have been documented. For instance, respiratory activity has been shown to affect expression of the nuclear-encoded mitochondrial alternative oxidase (Vanlerberghe and McIntosh, 1994), while chloroplast development seems to control expression of nuclear photosynthesis genes (Lopez-Juez *et al.*, 1998). Despite the clear evidence, components involved in organelle-nuclear communication have not yet been identified. Signalling between organelles in the plant cell is still an unknown field and only a few components have been studied. For example, exchange of metabolites between peroxisomes and mitochondria has been shown to occur during photorespiration (Oliver and McIntosh, 1995; Oliver and Raman, 1995; Raghavendra *et al.*, 1998) Mitochondrial gene expression is up-regulated in photosynthetically deficient mutants, (Hedtke *et al.*, 1999b) suggesting communication between chloroplasts and mitochondria. Moreover, deletions in the mitochondrial genome have been shown to affect chloroplast development and photosynthetic activity (Newton, 1995).

Organelles control the energy balance of the cell in response to changing environmental conditions. Therefore, homeostasis of the cell is dependent on organellar activity. Control of gene expression is a very important and efficient way of regulating the activity of the cell, but once the gene products are synthesised, the cell needs another level of regulation. This so-called post-translational regulation represents a quick and effective way of keeping the cell processes under control upon changing conditions (Leon *et al.*, 1998). Proteolysis and protein phosphorylation are some of the mechanisms used by the cell to control protein activity. The importance of protein phosphorylation in regulation of protein activity has been shown in all kinds of organisms, affecting the structure, activity and affinity of specific proteins. Of special interest for this work is the study of a mitochondrial nucleoside diphosphate kinase (mtNDPK), an auto-phosphorylated protein, first reported in plants (pea) by Struglics and Håkansson (1998).

The aim of this work is to study some aspects of signalling in pea mitochondria, especially concerning regulation of mitochondrial gene expression and the potential role of the mtNDPK as one of the components of intracellular signalling in higher plants.

## THE PLANT MITOCHONDRION

### Origin

Recent studies have confirmed the monophyletic origin of mitochondria from an  $\alpha$ -proteobacterial (eubacterial) ancestor. The ancestor has its closest relatives in the rickettsial subgroup, that includes obligate intracellular parasites such as *Rickettsia*, *Ehrlichia* and *Anaplasma* (Andersson *et al.*, 1998; Sicheritz-Ponten *et al.*, 1998).

The endosymbiotic theory was until very recently the most accepted hypothesis explaining the mitochondrial origin. According to this theory, an eubacterial-aerobic endosymbiont invaded a host cell, thereby establishing a symbiotic relationship that was the origin of the eukaryotic cell. In this model, the host cell is believed to have been an early eukaryote, related to archaeobacteria, containing a nucleus but lacking mitochondria. Genetic analysis of the eukaryotic nucleus has revealed incorporation of part of the eubacterial genome. This incorporation is explained by the endosymbiotic theory as the result of gene transfer from the mitochondrion to the nucleus during evolution.

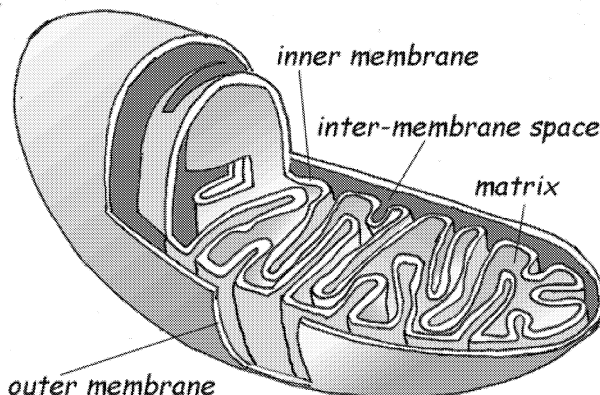
The acceptance of the endosymbiotic theory was challenged when analysis of genomes of *Archezoa*, a group of protists that lack mitochondria, showed the presence of genes of eubacterial origin (i.e. chaperonins) in the *Archezoa* nucleus (Bui *et al.*, 1996). This kind of evidence supports the “hydrogen hypothesis” (Martin and Muller, 1998) that proposes a simultaneous origin of the mitochondrion and the nucleus. It explains the origin of the eukaryotic cell as the result of a fusion event that occurred between a hydrogen-requiring archaeobacterium and a hydrogen producing eubacterium ( $\alpha$ -proteobacterium). In the hydrogen hypothesis, the reduction of the eubacterial genome results in the mitochondrial genome

and the combination of archae- and eubacterial genes gives the nuclear genome.

It may be possible to explain the origin of mitochondria by combining the two hypotheses. The fusion event explains the chimeric nature of the nucleus but does not exclude the possibility of a subsequent endosymbiotic event that gave rise to mitochondria (Gray *et al.*, 1999). The question then lies in whether the mitochondrion originated from an endosymbiotic event posterior to the formation of the nucleus or from a simultaneous event, that gave rise to both the mitochondrion and the nucleus.

## Structure

The mitochondrion (figure 1) has two membranes, the outer and the inner membranes. The presence of porin, a channel protein, makes the outer membrane permeable to molecules with a size smaller than 10 kDa. In contrast, the inner membrane is impermeable to most small solute particles, including protons. Together these membranes form two compartments, the inter-membrane space (between the two membranes) and the matrix, surrounded by the inner membrane.



**Figure 1.** Membranes and compartments of the mitochondrion (redrawn from Wolfe, 1993).

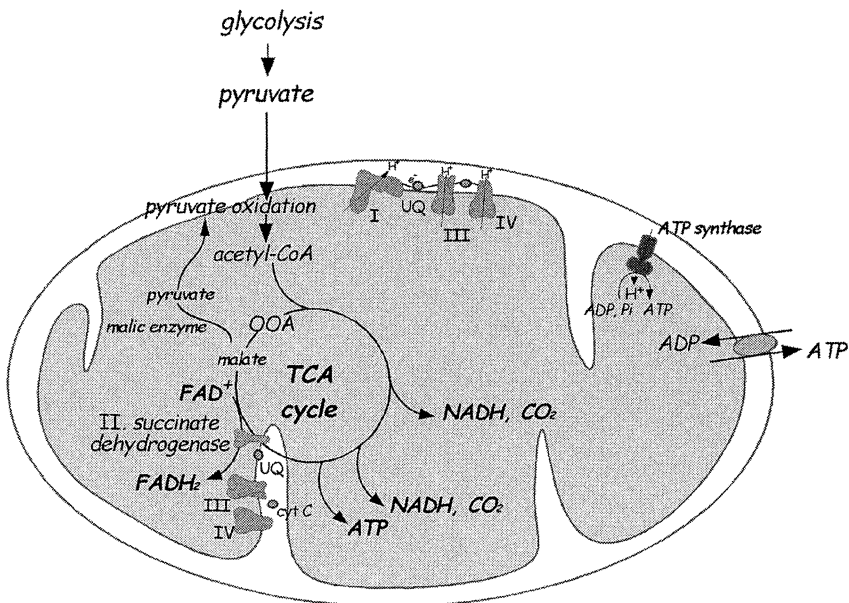


The inner membrane contains protein complexes responsible for respiration (the respiratory chain), ATP production, and transport of substrates into the matrix. The inner membrane is folded, forming numerous cristae that increase surface area of the membrane.

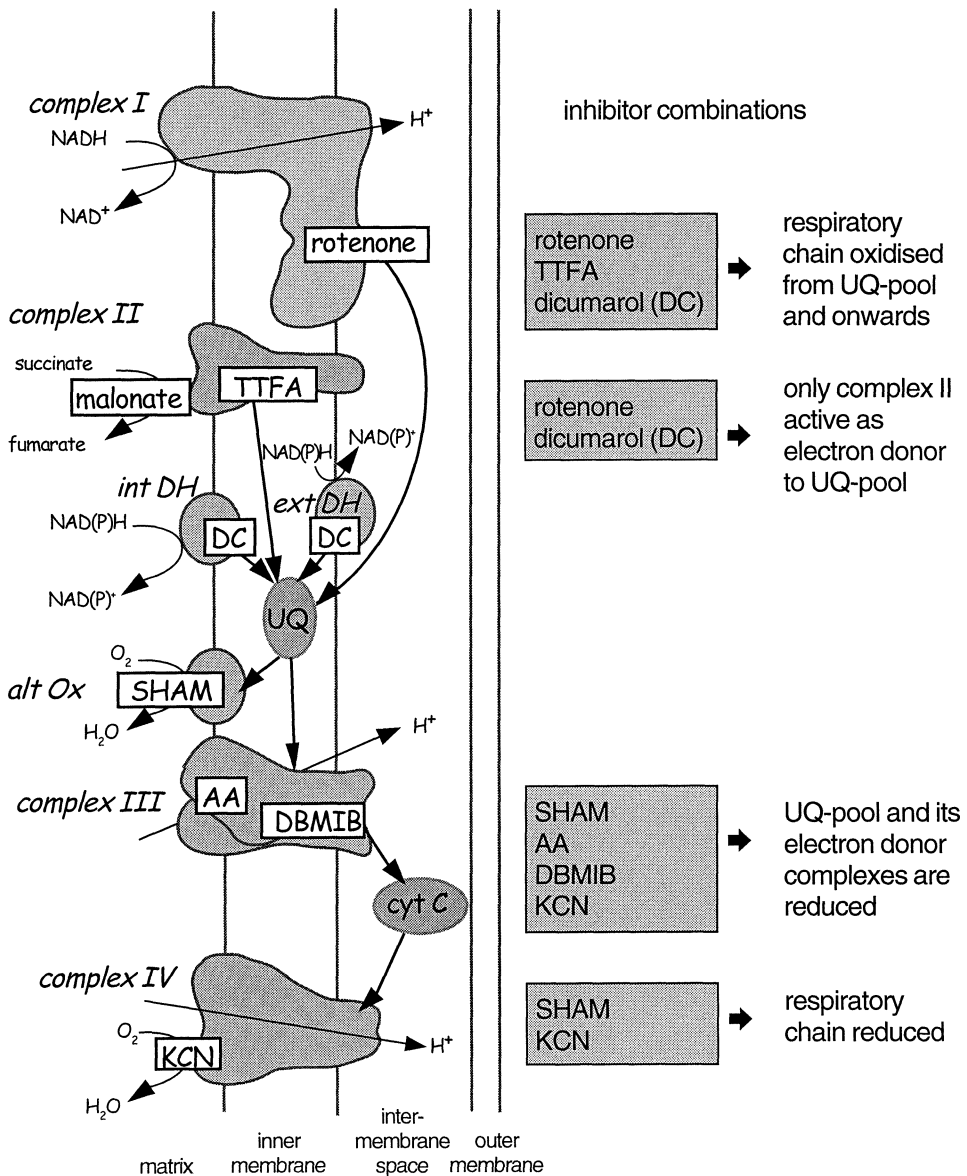
## Physiology

### The TCA cycle

The tricarboxylic cycle (TCA cycle) takes place in the mitochondrial matrix (figure 2). In the TCA cycle, carbon compounds are oxidised producing NADH, FADH<sub>2</sub> and CO<sub>2</sub>. The CO<sub>2</sub> is a waste product while the NADH and FADH<sub>2</sub>, molecules carrying electrons at low electrochemical potential, are oxidised by the components of the respiratory chain.



**Figure 2.** Some of the metabolic reactions that occur in plant mitochondria. OOA, oxaloacetate; UQ, ubiquinone pool; cyt C, cytochrome c. I-IV represent respiratory complexes I-IV, respectively.



**Figure 3.** Schematic representation of the plant mitochondrial respiratory chain including inhibitors used in Paper I and Paper II. Represented as int DH and ext DH, the internal and external alternative NAD(P)H dehydrogenases, respectively; UQ, the ubiquinone pool; alt Ox, the alternative oxidase; cyt c, cytochrome c. For practical reasons int DH and ext DH represent two distinct complexes in each case. The sites of action of inhibitors are represented by white boxes. Redrawn from the original picture, a kind gift of Dr. Allan Rasmusson.

The cycle starts when acetyl-CoA, produced by pyruvate oxidation, reacts with oxaloacetate producing citric acid. The release of two molecules of CO<sub>2</sub> regenerates the oxaloacetate and the cycle can start again. The FAD group is part of the succinate dehydrogenase in the respiratory chain, which transfers electrons to the ubiquinone pool.

### ***The respiratory chain***

The respiratory electron transport chain consists of four major complexes, I-IV (figure 3). Complex I and II oxidise NADH and succinate, respectively, reducing the ubiquinone pool. The electrons are then transferred from the ubiquinone pool to cytochrome c through complex III. The reduced cytochrome c is oxidised by complex IV, which then reduces oxygen to water. As electrons flow through the respiratory chain protons are pumped from the matrix to the inter-membrane space. This generates an electrochemical proton gradient across the inner mitochondrial membrane, because of the impermeability of the inner membrane to protons. The electrochemical proton gradient can be dissipated by the transfer of protons, back to the matrix, the by the proton-translocating ATP synthase, which then synthesises ATP from ADP and Pi. Electron transport sometimes can occur without the generation of a proton gradient, and thus respiration is uncoupled from ATP production. In plants, this uncoupling is achieved by the presence of additional electron transfer complexes responsible for the oxidation of NADH and NADPH, the alternative NAD(P)H dehydrogenases, as well as electron transfer from the ubiquinone pool directly to oxygen through an alternative oxidase.

## **Genome**

### ***Size***

Mitochondrial genomes vary greatly in size between organisms, exemplified by the relative small animal genomes (15-16 kb) and the large plant ones (180-2400 kb) (Wolstenholme and Fauron, 1995). Plant mitochondrial genomes are complex and dynamic entities. Events such as

homologous recombination and incorporation of foreign DNA have contributed to this complexity.

### ***Organisation***

The plant mitochondrial genome is believed to be organised in one master circle plus a set of subgenomic circles (for references see Wolstenholme and Fauron, 1995). The entire mitochondrial DNA is contained in the master circle and the smaller circles seem to be the products of recombinations between repeated sequences in the master circle. In addition to the mitochondrial genome, extragenomic DNA is commonly found organised in circular and linear plasmids. Less common are the self-replicating RNA plasmids found in maize (Finnegan and Brown, 1986).

### ***Coding capacity***

Mitochondrial genome contents are highly conserved among organisms. The mitochondrial genome encodes components of the respiratory complexes, ribosomal proteins, transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) and a small set of other proteins. The mitochondrial genome of *Arabidopsis thaliana* was the first genome to be completely analysed in angiosperms (Unsold *et al.*, 1997). The 367 kb genome contains 57 genes (summarised in table I) and 85 additional open reading frames, while about 60% of the DNA has no apparent function. Recently, the nucleotide sequence of the sugar beet mitochondrial genome was also determined (Kubo *et al.*, 2000). The sugar beet mitochondrial genome shares characteristics with the *Arabidopsis* genome having 59 genes and with 55.6% of the DNA lacking obvious function.

## **Mitochondrial gene expression**

### ***Promoters***

The presence of several different promoters in the mitochondrial genome has been experimentally demonstrated in higher plants (Bergman *et al.*, 1995; Binder and Brennicke, 1993; Brown *et al.*, 1991; Covello and Gray, 1991). Multiple promoters for some of the mitochondrial genes have also

been found (Mulligan *et al.*, 1988; Newton *et al.*, 1995; Tracy and Stern, 1995) and differential activity of these promoters might be an important factor in regulation of mitochondrial transcription (Binder *et al.*, 1996).

**Table I.** *Arabidopsis thaliana* mitochondrial DNA-encoded genes, adapted from Udseld *et al.* (1997) and Giegé *et al.* (1998).

product	genes
<i>respiratory chain components</i>	
complex I	<i>nad1, nad2, nad3, nad4, nad4L, nad5, nad6, nad7 &amp; nad9</i>
complex II	<i>sdh4</i> , edited transcript possibly translated
complex III	<i>cob</i>
complex IV	<i>cox1, cox2 &amp; cox3</i>
ATP-synthase complex	<i>atp1, atp6 &amp; atp9</i>
cytochrome-c-biogenesis	<i>ccb206, ccb256, ccb452, ccb382 &amp; ccb203</i>
<i>ribosomal proteins</i>	
large subunit	<i>rpl2, rpl5 &amp; rpl16</i>
small subunit	<i>rps3, rps4, rps7 &amp; rps12</i>
<i>ribosomal RNAs</i>	<i>rm5, rrn18 &amp; rrn26</i>
<i>transfer RNAs</i>	22, lacking tRNAs for 6 amino acids
<i>other ORFs</i>	<i>orfX, orfB &amp; orf25</i>

Inspection of the transcription initiation sites in monocot plants has revealed the presence of a well conserved CRTA (R=A/G) motif together with an A-rich upstream region that enhances transcription. The CRTA motifs are also found in dicots but in those it is part of a longer motif CRTAAGAGA (Binder *et al.*, 1996). Studies in pea (Binder *et al.*, 1995) show that the conserved motif alone is insufficient for transcriptional

activity, defining a 25 nucleotides region that includes the nonanucleotide motif plus an upstream A/T-rich region. However, the presence of transcription initiation sites that lack consensus sequences has also been documented, these being more commonly found in dicots than in monocots (for examples see Fey & Maréchal-Drouard, 1999).

### ***DNA-binding proteins***

Hatzack *et al.* (1998) identified two proteins (32 and 44 kDa) that bind to the promoter regions of *atp9* in pea and *atp1* in *Oenothera berteriana*. No similarity was found with DNA-binding proteins that are involved in initiation of mitochondrial transcription in humans and yeast, although the presence of the 32 and 44 kDa proteins enhances *in vitro* transcription assays carried out in plants.

### ***Transcription***

Transcription in plant mitochondria is carried out by a phage type RNA polymerase. This nuclear-encoded polymerase resembles the RNA polymerases of bacteriophages T3, T7 and SP6 (Hedtke *et al.*, 1997; Hedtke *et al.*, 1999a). Control of transcriptional activity has been proposed to be mainly related to promoter activity and regulation of the steady-state levels of RNA (Gray and Wong, 1992; Mulligan *et al.*, 1988).

### ***Translation***

Studies of translation in plant mitochondria have not been very successful, due to the lack of efficient *in vitro* systems. In spite of this, evidence of conserved blocks in the 5' untranslated region of mitochondrial mRNAs suggests a possible role for these elements in translational control. So far, however, there is only fragmented evidence of regulation of translation (Brown, 1998). Studies in cytoplasmic male sterile (CMS) lines in maize and *Petunia*, showed that restorer nuclear genes, necessary for recovery of fertility, might be involved in translational control (Kennell and Pring, 1989; Pruitt and Hanson, 1991). Editing makes mRNAs translation-competent (Grohmann *et al.*, 1994; Phreaner *et al.*, 1996) and in some cases allows the formation of initiation codons, representing another possible regulatory point in plant mitochondrial gene expression.

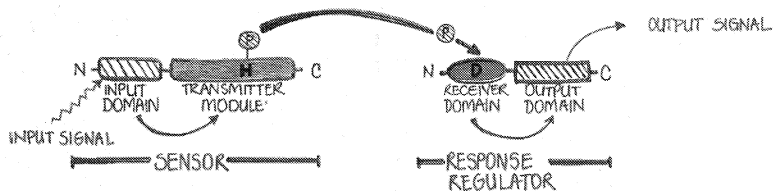
## REGULATION OF MITOCHONDRIAL GENE EXPRESSION

### Developmental regulation

Expression of mitochondrial genes is stimulated during processes whose energy requirements are high (Smart *et al.*, 1994). Events like anther and pollen development accompany increased transcriptional activity. This increase applies not only to mitochondrial genes but also to nuclear-encoded mitochondrial genes (Brennicke *et al.*, 1999). Many other studies have shown tissue and developmental differences in transcriptional levels of nuclear-encoded mitochondrial proteins. Landschütze and co-workers (1995a) showed that citrate synthase was found to be especially abundant in green flower buds and mature leaves in potato. Summarised in Rasmusson *et al.* (1998), studies on expression of nuclear genes encoding complex I also revealed higher levels of expression in flowers than in leaf and root/tuber. In maize, transcripts of the  $E_1\alpha$  and  $E_1\beta$  subunits of the pyruvate dehydrogenase were found to be more abundant in roots than in leaves or etiolated shoots (Thelen *et al.*, 1999).

### Environmental regulation

In bacteria, expression of a large number of genes has been shown to be controlled by two-component regulatory systems, in response to changes in environmental conditions (for a review see Parkinson and Kofoid, 1992).



**Figure 4.** Simplified representation of the bacterial two-component systems. Conserved regions represented by solid boxes. Redrawn from Parkinson & Kofoid, 1992.

Two-component systems consist of a "sensor" and a "response regulator" (figure 4). The so-called sensor is usually membrane-bound and undergoes auto-phosphorylation on a histidine residue upon specific changes in the environment. The phosphate group is then transferred from the histidine to an aspartate residue on the response regulator. The response regulator is often a DNA-binding protein whose activity is regulated by the presence or absence of a phosphate group on the aspartate residue.

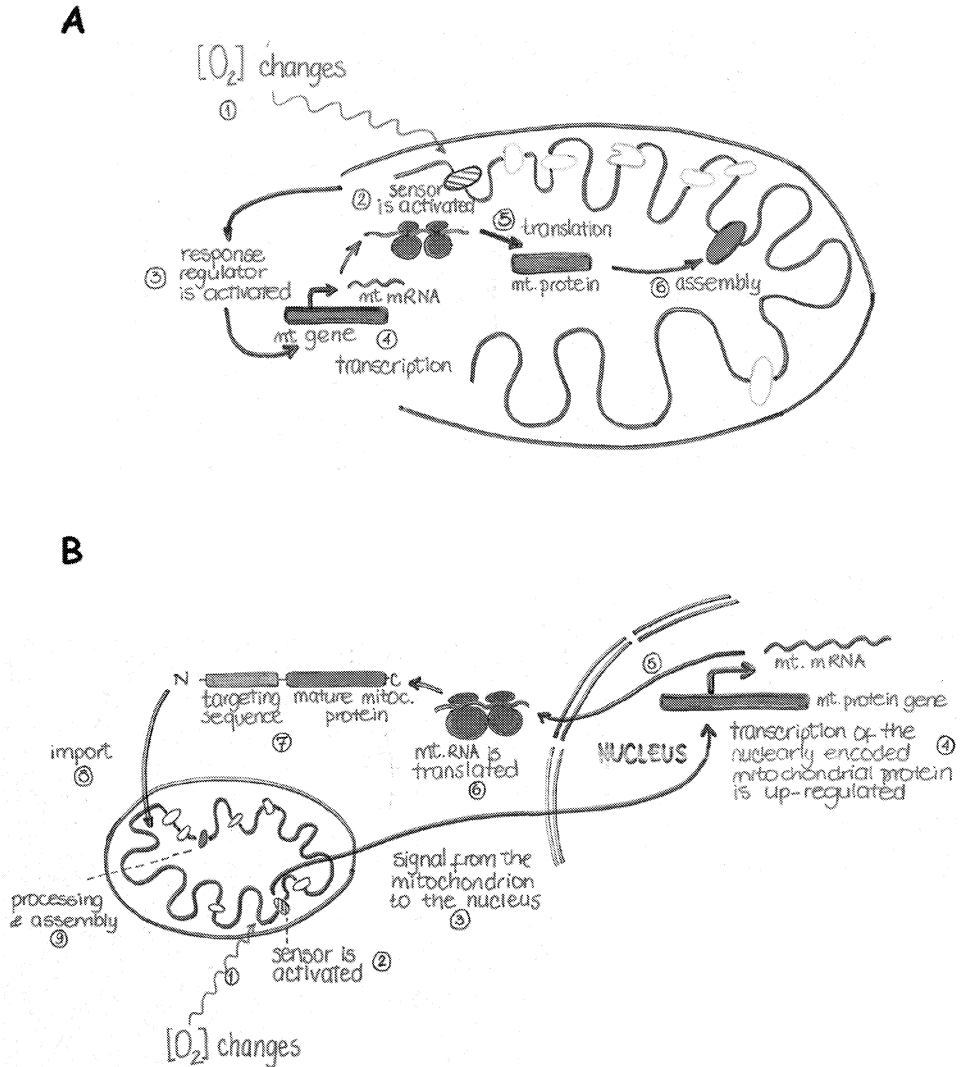
### ***Redox regulation***

Because of the homology of chloroplasts and mitochondria to bacterial systems, the presence of two-component systems in these organelles has been proposed as a possible mechanism for responses to changes in the environment (Allen, 1993a). Fluctuation in the availability of light and/or oxygen causes changes in the redox potential of the components of the organellar electron transport chains. The presence of a "redox sensor", associated with a component of the electron transport chain, would allow the organelle to initiate control of gene expression by activation of a "redox response regulator" (Allen, 1993b). Such redox regulatory systems are known in bacteria. For example in *E. coli*, the Arc (anoxic redox control, previously named aerobic respiration control) system (Iuchi and Lin, 1988), controls expression of a large set of genes. The system consists of the redox sensor ArcB and the cytoplasmic response regulator ArcA. ArcB is a transmembrane kinase with two transmitter domains and a receiver domain (Georgellis *et al.*, 1999). ArcB is believed to interact with components of the respiratory chain (Iuchi *et al.*, 1990) detecting changes in redox poise. Phosphorylation of ArcA is then catalysed by ArcB, repressing gene expression of about 30 operones upon anaerobiosis.

In spite of the continuous transfer of mitochondrial and chloroplast genes to the nucleus (as an example see Figueroa *et al.*, 1999) there is a set of genes that persist as the organellar genomes. One way to explain the presence of the mitochondrial and chloroplast genomes is the need for a rapid regulation of their genes upon changes in the environment that can be sensed by the organelle. A model that explains the differences between a nuclear and a mitochondrial response to a changing environmental stimulus is shown in figure 5. In panel A, the presence of a redox regulatory system allows a rapid response from the organellar genetic



machinery to changes in oxygen concentration. In contrast, a model for the response to oxygen concentration changes by the nuclear machinery is depicted in panel B.



**Figure 5.** Schematic representation of a mitochondrial (A) vs a nuclear (B) localisation for genes, whose expression is dependent on environmental changes

The second model must include a regulatory system that transfers the signal from the mitochondrion to the nucleus as well as a targeting sequence and translocation apparatus for correct sorting of the protein into the mitochondrion. The main difference between the two models would be the speed of the response. The maintenance of the mitochondrial genes would represent a better choice for the homeostasis of the cell when a rapid response is required.

Redox regulation of organellar gene expression has so far mainly been studied in chloroplasts. By the use of specific electron transport inhibitors, Pearson *et al.* (1993) showed that RNA synthesis in isolated chloroplasts is favoured by oxidation of the cytochrome *b<sub>6</sub>f* complex. Chloroplast mRNA stability in pea was shown to be redox dependent *in vitro* (Alexciev and Tullberg, 1997); the *petB* transcript amounts decreased under oxidising conditions while reducing conditions did not show any effect on the stability of the transcript. In mustard, transcription of the *psaAB* genes, that encode the two major subunits of the photosystem I reaction centre, is regulated by the redox poise of plastoquinone (Pfannschmidt *et al.*, 1999a). When plastoquinone becomes reduced, as a product of illumination with photosystem II (PSII) light, *psaAB* genes are up-regulated. In contrast, the *psbA* gene, encoding the D<sub>1</sub> reaction centre protein of the photosystem II is down-regulated under the same conditions. Using the same system to study the expression of other chloroplast genes did not show differences upon changes in light quality except for the *rbcL* gene, that is regulated in the same way as *psbA* (Pfannschmidt *et al.*, 1999b). Pfannschmidt and co-workers concluded from this study that the regulation of transcription of *psaAB* and *psbA* genes upon changing light regimes is gene-specific.

Danon and Mayfield (1994) showed redox regulation of the translation of chloroplast *psbA*-messenger RNA in *Chlamydomonas reinhardtii*, *in vitro*. Translation of the mRNA requires binding of nuclear encoded translational activators. Both translation and binding were found to be inhibited under oxidising conditions (Danon and Mayfield, 1994). Furthermore, changes in protein synthesis in response to changes in the redox potential have been shown in isolated pea chloroplasts (Allen *et al.*, 1995).

In comparison with the many examples of redox regulation in chloroplasts, there are few examples in plant mitochondria. With the use of specific respiratory inhibitors in isolated potato mitochondria, Wilson *et al.* (1996) showed that radiolabelled UTP incorporation into RNA was regulated by the redox poise of the Rieske iron-sulphur protein. Using a similar approach, redox regulation of mitochondrial gene expression has been shown in pea (Allen *et al.*, 1995), a phenomenon that has been further characterised in the work described in *Papers I and II*.

The redox state of mitochondria is also important for expression of nuclear genes encoding mitochondrial components. In *Nicotiana tabacum*, inhibition of electron transport at the site of complex III by addition of antimycin A has been shown to result in an increase of the expression of the nuclear gene *Aox1*, which encodes the alternative oxidase of plant mitochondria (Vanlerberghe and McIntosh, 1994). In an analogous situation, expression of the nuclear *cab* genes (coding for polypeptides of the light harvesting complexes), in green algae, has been reported to be redox regulated (Escoubas *et al.*, 1995). Redox control of the *cab* genes is exerted by the redox poise of the plastoquinone pool in chloroplasts.

***The expression of pea mitochondrial genes is regulated by the redox state of respiratory complex II (Paper I & II)***

*In organello translation*

By the use of specific inhibitors of the electron respiratory chain (see figure 3 for inhibitors) we have been able to show that the redox state of the succinate:ubiquinone oxidoreductase (complex II) plays a key role in the regulation of mitochondrial translation (*Paper I*). The incorporation of <sup>35</sup>S-methionine into newly synthesised mitochondrial proteins was shown to be dependent on the electron flux through the respiratory complex II, but was independent of the role of this complex in the TCA cycle.

Addition of thenoyltrifluoroacetone (TTFA) or malonate, two chemically unrelated inhibitors of complex II, inhibited protein synthesis in isolated mitochondria (*Paper I, figure 4, lane 4 & figure 6, lane 3*). However, none of the other specific respiratory complex inhibitors used in the study (table II) had a comparable effect on <sup>35</sup>S-methionine incorporation.

**Table II.** Effect on *in organello* translation of changes in the activity of mitochondrial respiratory complexes

Effect	respiratory complex(es) inhibited
inhibition	complex II complex I, II & alternative dehydrogenases
increase	complex I & alternative dehydrogenases
no change	complex I alternative dehydrogenases complex III complex IV alternative oxidase complex IV & III complex III & alternative oxidase

Since inhibition of complex II also inhibits the TCA cycle, it was important to see whether the observed effects on protein synthesis were the result of TCA cycle inhibition or of changes in the redox state of complex II. In order to distinguish between these two possibilities, we used two different approaches to inactivate the TCA cycle while keeping a reduced complex II. The first experiment consisted of depletion of endogenous substrates present in the mitochondria by incubation of the mitochondria at 25°C for 90 minutes. The treatment causes inhibition of the TCA cycle and oxidation of the respiratory chain due to lack of substrates. This results in inhibition of protein synthesis. Subsequent addition of succinate initiated electron transport through complex II but was not sufficient to start the TCA cycle, due to depletion of additional TCA-cycle intermediates. The addition of succinate supported protein synthesis while addition of NADH had no such effect even at amounts sufficient to support respiration (*Paper I, figure 5*). These results were consistent with the effects observed when

inhibitors were used, demonstrating that electron transport through complex II is necessary for mitochondrial translation. Thus, we could show that the observed effects were not merely the result of a general chemical influence on the system, caused by the presence of the inhibitors. Furthermore, the use of malonate together with rotenone, salicylhydroxamic acid (SHAM) and antimycin A in the presence of NADH (and calcium) causes a reduction of complex II at the same time as the TCA cycle is inhibited (*Paper I, figure 6*). NADH is used by the external NADH dehydrogenase, reducing the UQ-pool; however, its oxidation by complex III and/or the alternative oxidase, is prevented by the presence of antimycin A and SHAM. This situation allows back-flow of electrons from the UQ-pool to complex II. Presence of malonate “traps” the electrons in complex II, causing its reduction. Under these conditions protein synthesis can be sustained, showing that the electron flow through complex II is enough to support <sup>35</sup>S-methionine incorporation into newly synthesised mitochondrial proteins.

Our results support the hypothesis that proposes the presence of a redox regulatory system in pea mitochondria (Allen, 1993a). Furthermore, we show that the point of regulation is the succinate dehydrogenase complex. The presence of this regulatory system enables the mitochondrion to couple the TCA cycle activity to the synthesis of new components of the respiratory chain. If TCA cycle activity exceeds the capacity of the respiratory chain, mitochondrial protein synthesis must be up-regulated. For example, lack of subunits of complex I, III and IV when TCA cycle activity is high, will cause reduction of complex II. A reduced complex II would then support protein synthesis. This situation can be mimicked by the presence of two inhibitors, rotenone and dicumarol, that prevent transfer of electrons through the NAD(P)H dehydrogenases, leaving complex II as the only electron donor to the ubiquinone pool. The presence of rotenone and dicumarol enhances protein synthesis as the result of increase in activity of complex II (*Paper I, figure 4, lane 5*). Therefore, once the production of reducing power (NADH, FADH<sub>2</sub>) by TCA cycle exceeds the capacity of the respiratory chain, synthesis of new components needs to be increased.

As mentioned in *Paper I*, redox titration was suggested to be an appropriate approach to identify which one of the complex II components

is directly involved in the regulation of mitochondrial translation. Two procedures were used, neither of which seemed to be suitable for this purpose. No translation could be detected when the redox titration described by Allen and Holmes (1986) was carried out. This kind of titration requires the use of strong reducing and oxidising agents together with redox mediators under anaerobic conditions. It is likely that mitochondrial translation is abolished upon such treatment. On the other hand, no conclusive results were obtained when a less harsh method (Rich *et al.*, 1990) was tried. This method turned out to have lower reproducibility between assays and a more restricted range of possible redox potentials (only positive redox potentials could be obtained).

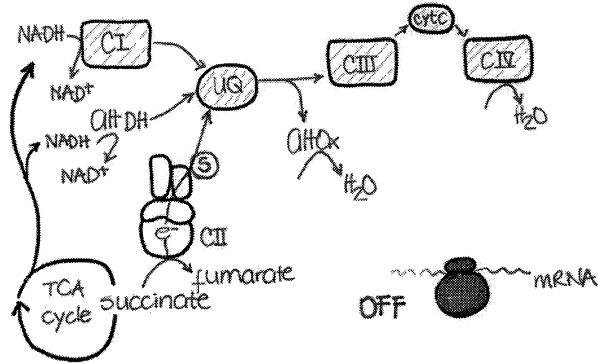
#### *Run-on transcription*

Once the presence of a redox regulatory system was established in pea mitochondria, using <sup>35</sup>S-methionine incorporation assays, it was necessary to define whether the regulation of gene expression was exerted on the transcriptional or the translational level. In run-on transcription assays, carried out using isolated and permeabilised pea mitochondria, partial inhibition of incorporation of <sup>32</sup>P-labelled UTP was achieved by the presence of TTFA (*Paper II, figure 1*). The decrease in incorporation is not comparable to the inhibition achieved in translation assays. This suggests that the activity of complex II regulates protein synthesis mainly at the translational level.

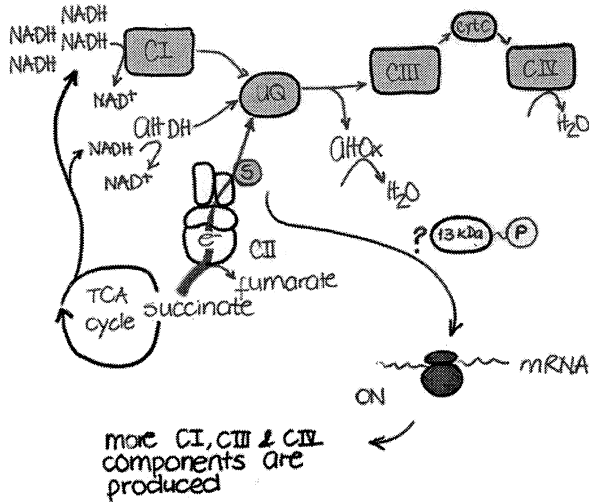
#### *Protein synthesis and phosphorylation*

In an attempt to elucidate the mechanisms behind the redox regulation of gene expression in mitochondria, protein phosphorylation assays were carried out. After *in organello* translation was performed, as described in paper I, mitochondria were incubated in the presence of radio-labelled ATP (*Paper II, figure 2*). The phosphorylation of a 13 kDa protein was induced by protein synthesis (*lane 2*) but inhibited by the presence of TTFA during the translation assay (*lane 3*). Thus, phosphorylation of the 13 kDa protein requires translational activity as well as an active complex II. Based on these observations, we proposed that phosphorylation of the 13 kDa protein is in some way correlated with the control that the redox state of complex II has on mitochondrial gene expression. Unfortunately, the minute amounts of the protein did not allow us to identify the 13 kDa protein.

"sensor" is oxidised



"sensor" is reduced



**Figure 6.** Hypothetical and simplified model for redox regulation of mitochondrial gene expression in plants. The "sensor" (S) becomes oxidised or reduced depending on the electron flow through complex II. Solid boxes represent "electron-saturated" components. Abbreviations as in figure 3, alt DH = int DH.

*A model?*

Summarising, a hypothetical model including the results from *Paper I* and *II*, is presented in figure 6.

## PHOSPHORYLATION AND SIGNALLING IN PLANT MITOCHONDRIA

Protein phosphorylation is one of the post-translational processes that enables the cell to control protein activity. It modifies protein structure by the addition of a phosphate group to specific amino acids (serine, threonine, tyrosine, histidine, aspartate), affecting not only protein structure but also protein function by means of factors such as affinity for substrates and oligomerisation. Phosphorylation is mediated by protein kinases, enzymes that catalyse the transfer of the phosphate group. Kinases are usually auto-phosphorylated in the presence of the phosphate donor (triphosphate nucleosides) and then transfer the phosphate group to their specific protein substrate. In plants, protein phosphorylation and auto-phosphorylation have been detected, that is protein kinase activity has been demonstrated in plant mitochondria (Miernyk and Randall, 1989; Struglics *et al.*, 2000; Struglics *et al.*, 1998; Struglics and Håkansson, 1999; Vidal *et al.*, 1993).

One of the most studied plant mitochondrial phosphoproteins is the E<sub>1</sub>α-subunit of the pyruvate dehydrogenase (PDH) complex (Miernyk and Randall, 1989). Phosphorylation of this subunit results in the inactivation of the complex while hydrolysis of the phosphate group causes its re-activation. So, activity of the complex is regulated by the activities of the kinase (inactivation) and the phosphatase (re-activation), this being a typical example of regulation of protein activity by reversible phosphorylation. Together with the E<sub>1</sub>α PDH subunit, another two soluble phosphoproteins have been identified in plant mitochondria, the α-subunit of the succinyl-CoA synthase (Oliver and McIntosh, 1995) and the mtHsp70 (Miernyk *et al.*, 1992). F<sub>1</sub>δ and F<sub>0</sub>b subunits of the ATP-synthase (Struglics *et al.*, 1998) have been identified as plant mitochondrial membrane phosphoproteins. Although membrane-associated mitochondrial kinase activity has been reported (Pical *et al.*, 1993; Struglics *et al.*, 2000), no such protein kinases have been identified.



Autophosphorylation, as mentioned before, is an intrinsic feature of protein kinases. A classical approach used to find autophosphorylated proteins is the use of ethylenediaminetetraacetic acid (EDTA) or ethylene glycol-bis-( $\beta$ -aminoethyl ether) (EGTA), chelators of divalent cations, in phosphorylation assays. Divalent cations, such as  $Mg^{2+}$  and  $Ca^{2+}$ , are necessary for protein kinase activity and, in their absence, only autophosphorylation can occur. When isolated pea mitochondria were phosphorylated in the presence of EDTA, a 17 kDa protein displayed autophosphorylation (Struglics and Håkansson, 1998; Struglics and Håkansson, 1999). The 17 kDa protein was identified as a nucleoside diphosphate kinase (NDPK).

### **Nucleoside diphosphate kinases**

Nucleoside diphosphate kinases (NDPKs) are enzymes that catalyse the transfer of phosphate groups between nucleoside phosphates. The reaction can be summarised as follows:



These enzymes are found in all kinds of organisms and have been mostly studied in animals. Animal NDPK isoforms, in addition to the enzymatic function described above, have been found to be involved in processes like control of cell proliferation (Cipollini *et al.*, 1997), regulation of transcription (Ji *et al.*, 1995; Postel *et al.*, 1993) and protein phosphotransferase activity (Engel *et al.*, 1998; Wagner and Vu, 2000). Although less is known in plants, the involvement of NDPK has been described UV-B light signalling (Zimmermann *et al.*, 1999), hormone responses (Nato *et al.*, 1997; Novikova *et al.*, 1999), and in phytochrome B response (Choi *et al.*, 1999),

### **The pea mitochondrial NDPK (*Paper III & IV*)**

Struglics and Håkansson (1999) characterised the pea mtNDPK biochemically. This soluble mitochondrial isoform was found to be

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† (Parks and Agarwal, 1973)

phosphorylated in the presence of EDTA. In the absence of divalent cations, transfer of the phosphate group to other substrates is prevented, trapping the phosphate on the NDPK. Since EDTA cannot reach the matrix of intact mitochondria, due to the impermeability of the inner membrane, it was concluded that the pea mtNDPK should be localised in the inter-membrane space. The pea mtNDPK is phosphorylated on histidine and serine, sharing these biochemical features with NM23-H1, a human NDPK isoform involved in signal transduction (McDonald *et al.*, 1993).

### ***Cloning of a cDNA encoding the pea mtNDPK***

Pea leaf cDNA was obtained by reverse transcription from total RNA and used as a template for PCR amplification. By the use of primers corresponding to the *N*-terminal sequence obtained by Struglics and Håkansson (1998) and a conserved region for all NDPKs, a 317 bp fragment of the pea mtNDPK cDNA was amplified. The fragment was labelled and used to screen a pea cDNA library allowing the isolation of a full length cDNA clone encoding the pea mtNDPK (*Paper III*).

### ***Sequence analysis and phylogeny of the mtNDPK***

The pea mtNDPK cDNA (figure 7) contains a 233 amino acids (aa) open reading frame (ORF). The ORF is preceded by 56 nucleotides (nt) and followed by 271 nt, corresponding to the 5' and 3' untranslated regions, respectively. The predicted amino acid translation of the ORF showed a *N*-terminal extension of 80 aa from the start of the protein, determined by sequencing of the purified protein (Struglics and Håkansson, 1998). The deduced amino acid sequence of the region of the clone corresponding to the mature protein, matched the partial sequences obtained previously (Struglics and Håkansson, 1998 and 1999).

Alignment of the pea mtNDPK with other NDPK isoforms (*Paper III*, figure 3A) allowed the construction of a phylogenetic tree (*Paper III*, figure 3B) in which five distinct groups can be observed. Interestingly these groups correspond to the subcellular localisations and origins of the examined NDPKs. It is clear that plant cytosolic isoforms group separately from their animal counterparts. The same is true for the mitochondrial

NDPKs, having clear differences between vertebrate and plant isoforms (pea mtNDPK and *Arabidopsis* NDPK3). The group designated vertebrate mitochondrial, contains three animal mitochondrial isoforms, the human Nm23-H4, a chicken and a pigeon isoforms.

```

1   GGC..ACG..AGA..CTC..ATG..CAC..GAC ATT CTG ACA TCA AGC TCA..TCG..ATA..CCG..TAG.
1   M   A   S   H   L   C   K   S   A   S   R   A   A   R   S   L
61  AAA ATG GCC TCA CAT CTC TGC AAA TCC GCT TCC AGA GCC GCT AGG TCT CTT
20  S   F   H   S   Q   G   R   A   V   A   A   A   A   A   V   A   S
121 TCT TTC CAC TCT CAA GGA CGC GCC GTA GCG GCG GCT GCA GCT GTT GCA TTC
40  V   P   V   F   A   P   N   Y   R   R   T   G   S   G   N   G   P
181 GTT CCG GTT TTT GCT CCA AAT TAC AGG AGA ACT GGT TCT GGA AAT GGA CCC
60  I   A   G   A   L   A   L   P   A   A   A   Y   M   L   Q   D   Q
241 ATT GCT GGA GCA CTC GCT CTT CCG GCT GCA GCT TAC ATG CTC CAA GAT CAG
80  A   ▼   A   E   L   E   R   T   F   I   A   I   K   P   D   G   V   Q
301 GCT GCC GAG CTG GAG CGC ACT TTC ATT GCC ATC AAG CCT GAC GGA GTG CAA
100 I   S   E   I   I   S   R   F   E   R   K   G   F   K   L   V   G
361 ATT TCA GAG ATT ATT TCT CGT TTC GAG AGG AAA GGG TTC AAG CTT GTG GGA
120 L   I   P   T   K   Q   F   A   Q   Q   H   Y   H   D   L   K   E
421 TTG ATT CCG ACA AAG CAA TTT GCC CAA CAG CAT TAT CAC GAC CTA AAA GAA
140 F   N   G   L   C   D   F   L   S   S   G   P   V   I   A   M   V
481 TTC AAT GGA TTG TGC GAC TTC CTG AGC TCT GGC CCT GTT ATT GCA ATG GTA
160 E   G   V   I   T   Y   G   R   K   L   I   G   A   T   D   P   Q
541 GAG GGA GTT ATT ACC TAC GGC CGT AAA CTA ATT GGA GCC ACA GAT CCA CAG
180 P   G   T   I   R   G   D   L   A   V   V   V   G   R   N   I   I
601 CCT GGA ACC ATT AGG GGT GAT CTG GCT GTT GTT GGA AGA AAT ATC ATC
200 D   G   P   E   T   A   K   D   E   I   K   L   W   F   K   P   E
661 GCA AAG GAT GAG ATA AAG TTG TGG TTT AAG CCA GAG GAA TTG GTT GAT GGT
220 S   F   T   S   N   S   E   K   W   I   Y   G   D   N   *
721 AGT TTC ACA AGC AAT TCA GAG AAG TGG ATT TAT GGC GAC AAC TGA ATT GGG
781 TCC TAC TTC ATT CTA CAT GAT TGA GTT TTC GAG AAA AAA TAA ATC ATT ATG
841 GGC AAT ACT GAA CTC TGC AAG CCA CCA ATA AGG GGC TGA GTA TAA TAA TGT
901 CAT GTC ATT CAG ATA GGA ATT GTA TTG TTT CAT TTT CTG TTT TCT TCA GTC
961 CAA TCT GCG ACA GTT TAT TTG TTT GAT GTT AAC AGT CAT TTT TCA ACA AAA
1021 CAT TTG GTT TTC CTA AAA AAA AAA AAA AAA AA

```

**Figure 7.** Nucleotide sequence of the pea mitochondrial NDPK and the deduced amino acid sequence. The sequences corresponding to the degenerated oligonucleotides used as primers are underlined. The N-terminus of the mature protein (determined by N-terminal sequencing, Struglics and Håkansson (1998)) is marked with an arrowhead. Dotted lines mark the 5'-untranslated region containing a putative stem-loop structure. The solid box indicates the putative polyadenylation site.

The last two isoforms are believed to be matrix located while recent work on the Nm23-H4 (Milon *et al.*, 2000) showed its association with mitochondrial inner and outer membranes, probably associated with their contact points. As expected, chloroplastic NDPKs group on their own, except for the spinach chloroplast isoform NDPK III, (Zhang *et al.*, 1995), which groups together with the plant mitochondrial NDPKs. In *Paper III* we proposed an inter-envelope localisation for the spinach NDPK III. This localisation would then be analogous to the inter-membrane space in mitochondria, which is believed to be the localisation for the pea and *Arabidopsis* mitochondrial NDPKs.

While *Paper III* was in press, two clones coding for Nm23-H6, a new human mitochondrial NDPK, were identified (Mehus *et al.*, 1999; Tsuiki *et al.*, 1999). It is important to mention that Tsuiki and co-workers found the Nm23-H6 clone by screening for molecules that suppressed induced apoptosis. Characterisation of Nm23-H6 (Tsuiki *et al.*, 1999) revealed the mitochondrial localisation of this protein as well as a putative role for this protein in control of mitosis.

### ***The pea mtNDPK is imported into mitochondria***

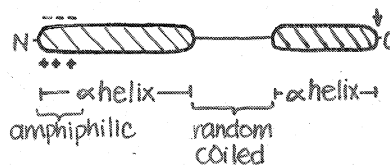
Although mitochondria have their own genome, most of the mitochondrial proteins are nuclear-encoded. After translation in the cytoplasm, transport into the mitochondria is carried out by a selective import machinery (for reviews see Braun and Schmitz, 1999; Glaser *et al.*, 1998; Rassow *et al.*, 1999). Signals in the sequence of proteins targeted to the mitochondria interact with components of the import apparatus. Often these signals are present as *N*-terminal extensions called pre-sequences or targeting sequences. Once the precursor protein is translocated into the mitochondrion, its pre-sequence is removed by a peptidase, yielding the mature form of the protein. The translocation of precursor proteins into the mitochondrion and their subsequent processing into mature proteins requires the presence of receptors, translocation machinery, processing peptidases and chaperones.

### ***Plant mitochondrial pre-sequences***

The presence of few acidic residues and a tendency to form amphiphilic  $\alpha$ -helices can be taken as common features of mitochondrial pre-

sequences among all organisms. Plant mitochondrial pre-sequences share some of the characteristics of mitochondrial pre-sequences found in animals and fungi, however plant pre-sequences are longer and contain more serine residues than their counterparts in other organisms (Sjöling and Glaser, 1998). In an analysis of known plant mitochondrial pre-sequences it was concluded that their *N*- and *C*-terminal regions are structurally more conserved than the central ones (Sjöling and Glaser, 1998).

An almost typical example of a mitochondrial pre-sequence is the 80 aa *N*-terminal extension of the pea mtNDPK (Figure 8; *Paper III, figure 1*). The first 17 aa form the amphiphilic  $\alpha$ -helix that makes the protein import-competent, whereas the central part has no predicted structure. The *C*-terminus of the pre-sequence is predicted to form another helix (figure 8), which in some cases has been shown to be necessary for recognition by the mitochondrial processing peptidase (MPP). However, the pea mtNDPK pre-sequence lacks the MPP recognition motif, an arginine at the -2 or -8 position from the cleavage site (Tanudji *et al.*, 1999).



**Figure 8.** Schematic representation of the pea mtNDPK pre-sequence. The cleavage site is indicated by an arrow.

#### *Translocation of nuclear-encoded mitochondrial proteins*

After interaction of the pre-sequence with receptors on the mitochondrial surface, translocation of the precursor protein into the mitochondrion can occur. Two proteins, 23 and 70 kDa, have been identified in plant mitochondria as import receptors by their similarity with the fungal receptors (Heins and Schmitz, 1996; Jansch *et al.*, 1998). Transport into the matrix, requires translocation of the precursors across the outer and the inner mitochondrial membranes. In the outer membrane, integral proteins involved in translocation are named TOM proteins while the inner

membrane counterparts are called TIM. TOM proteins have been characterised in potato (Jansch *et al.*, 1998), but less is known of the import machinery of the inner membrane. In *Arabidopsis thaliana*, two sequences encoding proteins similar to the fungal TIM proteins have been identified (Bömer *et al.*, 1996). The TIM proteins are believed to form a channel that allows the transit of precursors through the inner membrane, in a membrane potential dependent process (Dekker *et al.*, 1993). Once the precursor crosses the inner membrane, it interacts with the matrix heat shock protein mtHsp70 (Rassow *et al.*, 1994) and the nucleotide-exchange factor MGE (Braun and Schmitz, 1999). Binding of mtHsp70 to the *N*-terminus of the precursor is believed to be the driving force for protein translocation, in a reaction that requires ATP hydrolysis (Pfanner *et al.*, 1990).

Translocation of the pea mtNDPK precursor was inhibited by the presence of valinomycin during *in vitro* assays (*Paper III, figure 2*). This ionophore causes the dissipation of the mitochondrial membrane potential. The inhibitory effect of valinomycin on protein import, is an indication of the involvement of the inner membrane protein import machinery in the translocation of mitochondrial proteins (Hartl *et al.*, 1989).

### *Processing*

The correct assembly of the proteins, once their final destination is reached, requires cleavage of the pre-sequence. Most of the targeting sequences are removed by the action of the mitochondrial processing peptidase (MPP). In plants, MPP has been shown to be associated with the bc<sub>1</sub> complex (complex III) of the respiratory chain (Braun *et al.*, 1992a; Brumme *et al.*, 1998; Eriksson *et al.*, 1994). Although matrix-localised MPP activity has also been found (Szigyarto *et al.*, 1998), the matrix localised enzyme has not yet been purified. In fungi, precursors of proteins targeted to the inter-membrane space are processed by the inner membrane protease (IMP). Although experimental evidence has suggested the presence of IMP in plants (Braun *et al.*, 1992b), a plant IMP has not yet been identified.

Correct processing of *in vitro* translated products by the MPP can be achieved in isolated mitochondria by the addition of Triton X-100, which mildly solubilises the membrane (Whelan *et al.*, 1991). However, the pea

mtNDPK was not processed under these conditions, indicating an alternative processing system (*Paper III*). One possibility could be that the mtNDPK is processed by an IMP, which in analogy to the IMP found in thylakoids, recognises an alanine at position -1 from the first amino acid of the mature protein. Since the pea mtNDPK pre-sequence contains such an alanine, processing by an IMP remains a possibility, supporting the presence of an IMP in plant mitochondria.

#### *How does the pea mtNDPK reach the inter-membrane space?*

Two models for sorting of inter-membrane space proteins in mitochondria, both involving the inner membrane machinery, have been described in yeast mitochondria. The conservative model involves the complete translocation of the precursor into the matrix, removal of targeting sequence and export over the inner membrane to the inter-membrane space (for examples see Hartl *et al.*, 1989). The second one is the stop-transfer model, described by Bömer *et al.* (1997). After interaction of the precursor with the inner membrane protein import machinery the removal of matrix targeting sequence continues until it reaches a stop-signal in a hydrophobic helix, followed by lateral movement in the membrane. Based on our results, we can say that the import of the mtNDPK involves the inner membrane machinery (*Paper III, figure 2*). Therefore, any of the above described pathways might be feasible for the sorting of this mitochondrial protein into the inter-membrane space.

#### ***Differential expression of pea mtNDPK***

In green tissues, mitochondria must adapt to the changes in metabolism upon cell development. In non-photosynthetic tissues, mitochondria are the major source of ATP for the cell. In contrast, ATP production in photosynthetically active cells is shared with the chloroplasts and additional processes like photorespiration change the metabolic role of mitochondria. Using primary barley leaves, in which a developmental gradient can be found, Thompson *et al.* (1998) were able to show that differences in the amounts of mitochondrial proteins (mitochondrial and nuclear-encoded) were correlated with the degree of development of the studied cells. The results of this work suggested that changes in mitochondrial protein composition are the result of the change in mitochondrial function; in

young tissues mitochondria are energy suppliers while in mature tissues they are involved in other processes, such as photorespiration.

Through quantification of the mtNDPK transcripts, we concluded that the expression of the mtNDPK was higher in reproductive and young tissues as compared to vegetative and mature tissues (*Paper III, figure 4*). In agreement with these results, immunocytochemical detection of mtNDPK showed that this mitochondrial protein was more abundant in flowers and young leaves as compared to mature leaves (*Paper IV, figure 2*). Preferential localisation of the mtNDPK protein was observed in anthers, ovary and petals. Taking into account that in pea flowers, stamens and petals develop from the same primordia (Ferrandiz *et al.*, 1999), we could say that at this early stage in flower development the mtNDPK seems to be preferentially localised in the reproductive parts of the flower. A similar case was reported by Smart *et al.* (1994) who showed specific localisation of the mitochondrial  $\alpha$ -subunit of the  $F_1$ -ATP synthase in meiotic cells of sunflower anthers. Processes with high energy demands, such as microspore development, have been correlated with an increase in mitochondrial replication (Warmke and Lee, 1978). Furthermore, vegetative parts of potato plants with decreased amounts of citrate synthase developed normally, whereas flower formation was severely affected, showing the importance of mitochondrial function in flower development (Landschütze *et al.*, 1995b). On the basis of these findings, it would be logical that mitochondrial proteins should be more abundant in reproductive tissues than in vegetative ones.

Moreover, non-expanded leaves showed higher amounts of pea mtNDPK transcripts than mature leaves. In addition, the mtNDPK was more abundant in lower, as compared to upper, mesophyll cells in young leaves. These findings are consistent with results obtained in barley (Thompson *et al.*, 1998). The lower mesophyll contains less chloroplasts than the upper one, making this part of the leaf more dependent on the mitochondrial activity. The lower mesophyll is then analogous to the basal part of the barley leaf in which mitochondrial proteins involved in bioenergetic activity are more abundant (Thompson *et al.*, 1998).

However, when the amounts of mtNDPK protein were compared between mitochondria isolated from the different tissues, no significant differences



could be observed among the different samples (*Paper IV, figure 1*). The discrepancy between the western analysis and the results obtained by northern and immunocytochemical analysis can be explained on the basis of differences in mitochondrial number in the various studied tissues. A variation in mitochondrion number between tissues would not be detected in western analysis, which is based on analysis of equal amounts of isolated mitochondria from each tissue.

### ***Stress responses and the pea mtNDPK***

In *Paper IV* we studied the expression of the mtNDPK upon high salt, cold, heat and oxidative stresses. Using western analysis we observed that no significant differences could be observed in the amounts of mtNDPK under the different conditions (except for a minor decrease that can be explained by increase in protease activity). We also tried to determine if the mtNDPK was newly synthesised upon stress. Pea leaves were supplied with <sup>35</sup>S-methionine and exposed to the same stress conditions as above. Antiserum against the pea mtNDPK was used to precipitate *de novo* synthesised proteins from crude mitochondrial preparations. No newly synthesised mtNDPK could be detected. However, we observed that under heat stress, a <sup>35</sup>S-methionine labelled 86 kDa protein co-precipitated with the mtNDPK. This observation allowed us to propose that an interaction between the 86 kDa protein and the mtNDPK is involved in heat-stress response. Previous work in sugarcane, also suggested the involvement of a NDPK isoform in heat response (Moisyadi *et al.*, 1994). This NDPK, present in the microsomal fraction, displayed increased phosphorylation upon heat-shock.

### ***Heat stress in plant mitochondria***

Although characterisation of several mitochondrial heat-shock proteins has been reported in various organisms (Banzet *et al.*, 1998; Downs and Heckathorn, 1998; Hartman *et al.*, 1992; Heckathorn *et al.*, 1998; Herrmann *et al.*, 1994; Lund *et al.*, 1998; Neumann *et al.*, 1993; Wood *et al.*, 1998) not very much is known about the mechanism of heat stress response in plant mitochondria. Studies in maize (Lund *et al.*, 1998), showed that in this thermotolerant species, Hsp22 seems to be the main component of the mitochondrial response to heat-stress. Hsp22 is constitutively expressed at low levels and up-regulated during heat stress,

suggesting a specific role for this protein in primary heat-stress response. Downs and Heckathorn (1998) reported protection of the respiratory complex I by a small heat shock protein (1mw-hsp) upon heat stress, in apple. Respiratory activity through complex I was significantly reduced upon heat stress. This reduction in complex I respiratory capacity was prevented by heat-acclimation of the fruits prior to the measurements or by addition of purified 1mw-hsp. Moreover, the presence of specific antibodies against the 1mw-hsp antagonised the effect of the acclimation.

#### *The 86 kDa protein is a novel protein*

Analysis of the sequence obtained from the 86 kDa protein showed no similarities with other proteins found in the available databases. Even though we have not identified the 86 kDa protein, we can say that its interaction with the pea mtNDPK is probably part of heat-stress response in plant mitochondria. The mechanism behind this interaction remains to be investigated.

#### *Is the mtNDPK interacting with other proteins?*

Examples of interaction of NDPKs with diverse proteins have been reported in animals and in plants (Choi *et al.*, 1999; Engel *et al.*, 1998; Otero, 1997). Seeking for proteins that interacted with the pea mtNDPK, a pea cDNA library was screened using the yeast-two-hybrid system. This method, developed by Fields and Song (1989), allows detection of protein-protein interactions *in vivo*. In yeast, *lacZ* transcription is controlled by the transcriptional activator GAL4. GAL4 can be separated in two functional domains, the activation domain (AD) and the DNA-binding domain (BD). Separated from each other, these domains are unable to activate *lacZ* transcription. A plasmid expressing a hybrid consisting of the protein of interest and GAL4 DNA-binding domain is used as “bait”. The “target”, is generally a plasmid expressing hybrids of the activation domain and a library of proteins. When these two vectors are co-transformed into yeast, the “bait” protein binds upstream of the reporter gene *lacZ*, and screens for “target” proteins. An interacting “target” brings the AD and BD domains into proximity, reconstituting GAL4, and activating *lacZ* transcription. Activation is detectable by X-gal assay.

We used two constructs as “bait” vectors; the first included the entire ORF of the pea mtNDPK (precursor) whereas the region encoding the mature protein was used for the second construct (mature). The entire ORF was used in order to find proteins interacting with targeting sequence of pea mtNDPK, since we were interested in the processing of the precursor of this mitochondrial protein.

**Table III.** Pea mtNDPK putative interacting proteins, detected using yeast-two-hybrid system.

“bait”	“target”
precursor	<ul style="list-style-type: none"> <li>• 60S ribosomal protein L9, pea (X65155)</li> <li>• 93% similar to 60S ribosomal protein L27A, <i>A. thaliana</i> (AC010796)</li> <li>• 88% similar to 40S ribosomal protein S20, <i>A. thaliana</i> (AL096860)</li> <li>• 81% similar to RuvB DNA helicase-like protein, <i>A. thaliana</i> (AL132965)</li> <li>• 73% similar to ripenin-like protein, soya (AF127110)</li> </ul>
mature	<ul style="list-style-type: none"> <li>• arginine decarboxylase, pea (Z37540)</li> <li>• 90% similar to histone H2B, cotton (AF025667)</li> </ul>

After screening of co-transformants (“bait” and cDNA library) using the X-gal assay, positives clones were isolated. Interactions were verified by co-transforming the isolated plasmids containing the positive “targets” and the respective “bait” vectors. Transformations with the positives clones alone, were also carried out to select for clones that could activate the marker gene without interaction with the “bait” vector. Sequences of true positive clones, encoding possible interacting proteins with precursor and mature forms of the pea mtNDPK were analysed against available databases. The results of the searches are summarised in table III. These are only preliminary results and biochemical assays are required to confirm true interactions. Nevertheless, our data could be taken as an indication of the versatile nature of the pea mtNDPK.

### *The mtNDPK can be found in various oligomeric states*

NDPK function and oligomerisation state have been shown to be correlated (Mesnildrey *et al.*, 1998; Mesnildrey *et al.*, 1997). The presence of various oligomeric states has been suggested as a property of these enzymes that influences their interactions with different types of substrates. Most eukaryotic NDPK isoforms that have been crystallised form hexamers. In these isoforms, the enzymatically active form is hexameric and is believed to interact only with nucleosides, whereas the structure of dimers or tetramers would allow the binding of larger substrates such as proteins. Stability of the hexameric conformation of NDPK isoforms has been shown to be dependent on the presence of a C-terminal YE motif and a very conserved proline (Webb *et al.*, 1995). The conserved proline is found at position 95 in the pea mtNDPK, but the C-terminal YE motif is missing (*Paper III, figure 3A*). Absence of this motif might lead to tetrameric conformations, like the ones found in prokaryotes. In view of the prokaryotic origin of the mitochondrion, one could expect prokaryotic features for the mitochondrial NDPK isoform, such as the formation of tetramers. Moreover, analysis of the sequence of the cDNA encoding for this protein showed the presence of prokaryotic regulatory motifs in the 5' untranslated region of the clone (Escobar Galvis *et al.*, unpublished results, see figure 7).

Using gel filtration, we were able to detect the presence of the mtNDPK in fractions corresponding to hexameric (~ 100 kDa), tetrameric (~ 60 kDa) and dimeric states (~ 30 kDa) (*Paper IV, figure 5*). Hexamerisation has also been reported for other organellar isoforms that lack the YE motif, such as the spinach NDPK II (Yang and Lamppa, 1996) and the human mitochondrial Nm23-H4, which was crystallised as a hexamer (Milon *et al.*, 2000). Thus, it seems that the presence of the C-terminal YE is not an absolute requirement for hexamer formation. On the other hand, the presence of the mtNDPK in the fraction around 100 kDa might as well be explained by the formation of a complex between the novel 86 kDa protein (described in the previous section) and the mtNDPK.

So far, we cannot say which oligomeric state, is the enzymatically active form of the pea mtNDPK *in vivo*. If one takes into account the possibility that different oligomeric states might be associated with diverse functions

of this enzyme, a deeper study of this aspect must be carried out to fully understand its functions.

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In an attempt to address some aspects of signalling in plant mitochondria, I have studied regulation of mitochondrial gene expression and investigated the function of a novel mitochondrial protein – the pea mtNDPK.

My co-workers and I have presented clear evidence for redox regulation of translation in isolated pea mitochondria. Moreover, we have shown that protein synthesis is dependent on the activity/redox state of respiratory complex II (*Paper I*). We believe that protein phosphorylation is part of a regulatory system that couples the redox state of complex II and mitochondrial protein synthesis and that the phosphorylation of a 13 kDa protein might be involved (*Paper II*). However, we have not been able to define the components involved in redox regulation of mitochondrial gene expression. Perhaps, when more information about plant genomes becomes available, sequence comparisons will allow the identification of components of this regulatory system, using the existing data from prokaryotic and fungal systems. I believe that this approach might help to investigate the mechanisms behind the regulation of mitochondrial translation in plants, a field that seems to have been, to some extent, overlooked.

Concerning the pea mtNDPK, our results have confirmed the mitochondrial localisation of this protein (*Paper III*). We also found that this NDPK isoform has an expression pattern similar to other mitochondrial proteins involved in the bioenergetic function of mitochondria (*Paper IV*). Using phylogenetic analysis of some NDPK isoforms, we were able to classify them according to their subcellular localisation and origin. One could conclude that, in spite of the great similarity in sequence between the NDPK family members, small differences in sequence might reflect functional specificity associated with subcellular localisation.

There are several aspects that need to be further investigated and might help to elucidate the role of the pea mtNDPK in signalling in plant mitochondria. The detection of an interaction of the pea mtNDPK with a novel 86 kDa protein, which is up-regulated upon heat-stress, gave us evidence for the involvement of this mitochondrial NDPK in heat-stress response (*Paper IV*). Furthermore, it seems likely that the pea mtNDPK is present in various oligomeric states, a characteristic that has been suggested to enable NDPK isoforms to interact with different substrates (*Paper IV*). Without identification of the 86 kDa protein, it is not possible to determine the nature of its interaction with the pea mtNDPK. Therefore, cloning and overexpression of this novel protein would be the logical approach to start to investigate the role of the pea mtNDPK-86 kDa protein interaction in heat response. In order to facilitate the study of these possible interactions, overexpression of the pea mtNDPK will also be necessary, as well as the construction of mutated and/or truncated forms. With these resources, discrimination between enzymatic activity and any other possible functions for the pea mtNDPK could be feasible. An interesting aspect to study will be to determine if the pea mtNDPK displays phospho-transferase activity, as it has been shown for other NDPK isoforms (Engel *et al.*, 1995; Wagner and Vu, 2000), especially using the 86 kDa protein as substrate upon heat stress.

## POPULÄRVETENSKAPLIG SAMMANFATTNING

En del av cellens funktioner utförs i de membranomslutna små "rum" i cellen som kallas organeller. En typ av organeller är mitokondrierna vars främsta funktion är att producera energi – ATP syntes. ATP produceras under en process kallad respiration. Respirationen sker i mitokondriens membran där elektroner transporteras mellan proteinkomplex (komplex I-IV) och elektron bärare (ubiquinone och cytokrom c) (figur 3). Under respirationen konsumeras syre, NAD(P)H och succinat.

Mitokondrierna härstammar från frilevande bakterier som inneslutits i en annan organism (Gray, 1993). Detta ledde till uppkomsten av en ny, mer komplex livsform. Under evolutionens gång har de flesta av mitokondriens gener förflyttats till värdorganismens kärna. Ett fåtal gener finns dock fortfarande kvar i mitokondrien. De flesta av mitokondriens proteiner produceras därför utanför mitokondrien i den s.k. cytoplasman och endast ett fåtal är syntetiserade i mitokondrien. För att hela uppsättningen av mitokondriella proteiner skall produceras måste således två genetiska system användas och dessutom koordineras. Varför har då inte alla mitokondriella gener flyttats till kärnan? En förklaring till detta kan vara att uttrycket av generna i mitokondriens genom är, och måste vara, kontrollerade av förändringar av förhållanden, t ex syrekoncentrationen, innuti mitokondrien. Det är känt att hos vissa bakterier uttrycks en del gener endast vid hög syrekoncentration (Iuchi and Lin, 1988). Komponenter i bakteriernas respiratoriska system kan känna av skillnader i syrekoncentrationen och justera genuttrycket i enlighet med de rådande förhållandena.

På liknande sätt anses förändringar i mitokondriens respiratoriska aktivitet kunna påverka uttrycket av de mitokondriella generna (Allen, 1993a). För att testa denna hypotes förändrade vi aktiviteten hos respirationens komponenter och studerade vilka effekter dessa förändringar hade på proteinsyntesen i mitokondrierna. Endast då aktiviteten av komplex II var förändrad, kunde vi se påverkan av mitokondriell proteinsyntes (*Paper I*). Hur kan komplex II-aktiviteten påverka mitokondriellt genuttryck och proteinsyntes? Kan komplex II kommunicera med komponenter av det mitokondriella genuttryckssystemet? Vi kan ännu inte besvara dessa

frågor, men tror att translation (ett av de nödvändiga stegen i proteinsyntesen) är kontrollerad av aktiviteten hos komplex II. Dessutom verkar denna reglering involvera fosforylering av ett oidentifierat protein (*Paper II*).

Proteinfosforylering innebär att en fosfatgrupp binds till ett protein. Denna fosfatgrupp förändrar proteinets egenskaper, aktiverar eller inaktiverar proteiner, och kan fungera som medel för kommunikation i cellen. Mitokondriellt nukleosid difosfat kinas (mtNDPK) är ett fosfoprotein vars funktioner ännu inte är fullständigt undersökta. Vi har identifierat den fullständiga genetiska information som kodar för mtNDPK i ärtor och visat också att proteinet importeras in i mitokondrien (*Paper III*). Vi konstaterade vidare att mtNDPK är mer förekommande i blommorna än i andra delar av växten (*Paper III & IV*). Vi fann också att mtNDPK interagerar med ett protein som nybildas under värmestress. Vi förmodar därför att ärt-mtNDPK har en roll i växtens reaktion på värmestress (*Paper IV*).



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A handwritten signature in black ink, appearing to read 'Jens', with a stylized flourish at the end.

Lund, August 2000

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## Protein synthesis by isolated pea mitochondria is dependent on the activity of respiratory complex II

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**Abstract** In isolated pea (*Pisum sativum* L.) mitochondria incorporation of <sup>35</sup>S-methionine into newly synthesised proteins was influenced by the presence of site-specific inhibitors of the respiratory electron-transport chain. These effects were not produced by changes in the rate of respiratory electron transport itself nor by changes in ATP concentration. Protein synthesis was inhibited by inhibitors of ubiquinone reduction but not by inhibitors of ubiquinol oxidation. By the use of additional inhibitors at specific sites of the respiratory chain, different oxidation-reduction states were obtained for the different complexes in the electron-transport chain. It was found that electron transport through succinate:ubiquinone oxidoreductase (respiratory complex II) was specifically required for protein synthesis, even when all the other conditions for protein synthesis were satisfied. We suggest that a subunit of complex II, or a component closely associated with complex II, is involved in a regulatory system that couples electron transport to protein synthesis.

**Key words** Mitochondria · Protein synthesis · Succinate dehydrogenase · Regulation of gene expression

### Introduction

Control of gene expression in response to changes in redox potential has been shown to occur in bacteria as well as in nuclear gene expression in eukaryotic systems (Iuchi and Lin 1993; Meyer et al. 1994; Mosley et al. 1994). This control allows cells optimally to exploit changing environmental conditions. In bacteria, two-component systems control gene expression by means of “redox sensors”, de-

fining as electron carriers that initiate control of gene expression in response to changes in redox potential, and “redox response regulators”, DNA-binding proteins that affect gene expression under the action of one or more redox sensors (Allen 1993a). In eukaryotic cells, environmental changes causing alterations in the redox potential of components of electron-transport chains will be rapidly detected in chloroplasts and mitochondria, organelles with their own genetic system. Taking into account the prokaryotic origin of these organelles (Palmer 1992), it is reasonable to expect that redox regulatory systems are also present in chloroplasts and mitochondria (Allen 1993b, c).

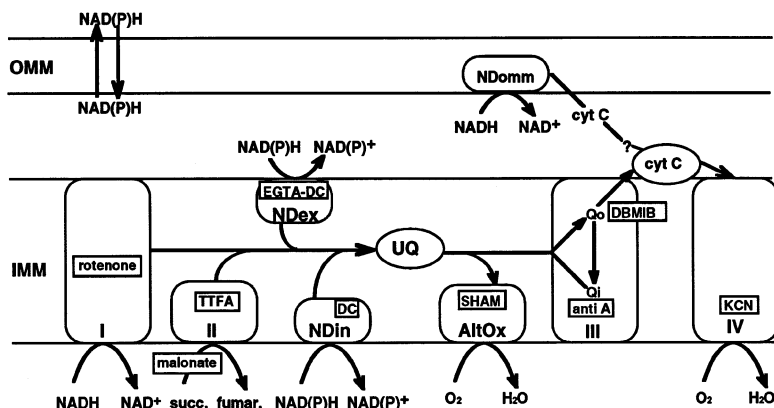
Results of recent investigations are consistent with redox regulation of organelle gene expression. By the use of specific electron-transport inhibitors Pearson et al. (1993) showed that RNA synthesis in isolated chloroplasts is favoured by oxidation of the cytochrome *b<sub>6</sub>f* complex. Using the same approach in isolated potato mitochondria Wilson et al. (1996) showed that radiolabelled UTP incorporation into RNA was regulated by the redox poise of the Rieske iron-sulphur protein. Danon and Mayfield (1994) showed redox regulation of the translation of chloroplast *psbA*-messenger RNAs in *Chlamydomonas reinhardtii* in vitro. Translation of these RNAs required binding of nuclear-encoded translational activators. Both translation and binding were found to be inhibited under oxidising conditions (Danon and Mayfield 1994). Furthermore, changes in protein synthesis in response to changes in the redox potential of the reaction medium have been demonstrated in isolated chloroplasts and mitochondria (Allen et al. 1995).

Besides regulating mitochondrial gene expression, the redox state of mitochondria is important for the expression of nuclear genes encoding mitochondrial components. In *Nicotiana tabacum* L., inhibition of electron-transport at the site of complex III by the addition of antimycin A has been shown to result in an increase of the expression of the nuclear gene *Aox1*, which encodes the alternative oxidase of plant mitochondria (Vanlerberghe and McIntosh 1994).

In the present report different respiratory chain inhibitors and respiratory substrates were used in order to alter

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**Fig. 1** Schematic presentation of the inner membrane of plant mitochondria and respiratory chain components. Electron transport from NAD(P)H to  $O_2$  involves three of the four multiprotein complexes (I, III and IV), which are located in the inner membrane. Complex II oxidises succinate (*succ.*) to fumarate (*fumar.*) transferring electrons to the UQ-pool and subsequent electron carriers. Electron flow within complex III involves the two centres of cytochrome b ( $Q_i$  and  $Q_o$ ). ATP synthesis by oxidative phosphorylation occurs by means of coupling of the electron flow through complexes I, III and IV to proton transport across the inner membrane. The alternative oxidase (*AltOx*) and NAD(P)H dehydrogenases [*NDin* (internal) and *NDex* (external)] are specific to plants. Respiratory inhibitors and their action sites are indicated. *Anti A* Antimycin A; *DC* dicumarol; *NDom* outer membrane NADH dehydrogenase; *TTFA* thenoyltrifluoroacetone. Adapted from Rasmussen (1994)

the redox potential of specific complexes in the electron-transport chain and analyse corresponding changes in protein-synthesis patterns. Figure 1 shows a schematic presentation of the mitochondrial membrane and components of the respiratory chain, indicating the site of action of the inhibitors used in these experiments.

## Materials and methods

**Mitochondrial isolation.** Mitochondria were isolated from young pea (*Pisum sativum* L.) leaves (11–12-days-old, grown at 20°C with a 12-h day) according to Boutry et al. (1984), with modifications according to Håkansson et al. (1988).

**Redox-regulated translation.** Protein synthesis in isolated mitochondria was performed mainly according to Allen et al. (1995). In 10-ml sterile tubes 80–100 µg of protein ( $\leq 5$  µl of mitochondrial suspension) were added to 100 µl of synthesis medium (5 mM  $KH_2PO_4$ , 2 mM GTPdNa, 0.4 M mannitol, 60 mM HEPES, 10 mM  $MgCl_2$ , 25 µM of each of the amino acids excluding methionine, 4 mM ADP(K) and 4 mM ATP, 1% bovine serum albumin, pH 7.0). Different respiratory substrates and electron-transport inhibitors were included according to Table 1. After 15 min of incubation at 25°C on a shaker, 20–30 µCi of  $^{35}S$ -methionine was added to each tube, and the tubes were incubated again for 75 min. To stop the translation, 350 µl of suspension medium (plus 10 mM methionine) was added and the samples transferred to microfuge tubes. After centrifugation for 4 minutes at 16 000 g in a microcentrifuge, the supernatants were removed and the pellets dissolved in 30 µl of electrophoresis

**Table 1** Concentrations of respiratory substrates and respiratory chain inhibitors used, unless otherwise stated in figure texts

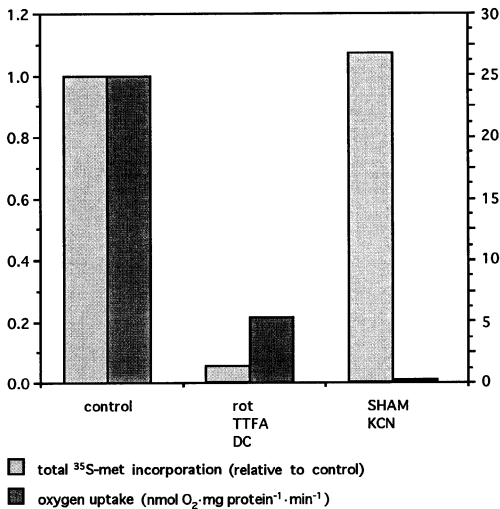
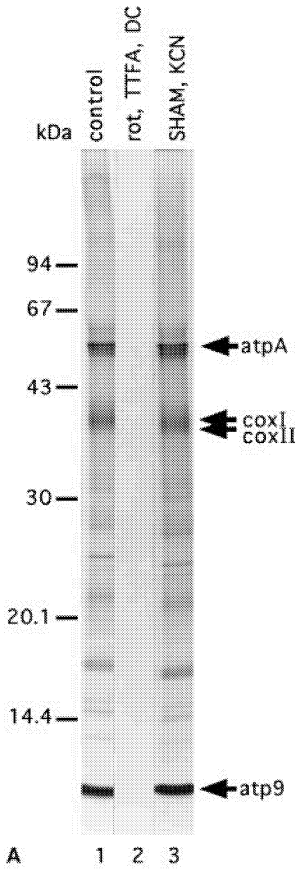
Common name	Final concentration	Solvent
Antimycin A	0.4 µM	H <sub>2</sub> O
CaCl <sub>2</sub>	1 mM	H <sub>2</sub> O
DBMIB	2 µM	Ethanol
Dicumarol	20 µM	DMSO
Duroquinol	0.1, 0.5 or 1 mM	Ethanol
KCN	1 mM	H <sub>2</sub> O
Malic acid	10 mM	H <sub>2</sub> O
Malonate	5 mM	H <sub>2</sub> O
Myxothiazol	15 µM	Ethanol
NADH	1 mM	H <sub>2</sub> O
Rotenone	50 µM	DMSO
SHAM	1 mM	DMSO
Na-pyruvate	1 mM	H <sub>2</sub> O
Succinate	5 mM	H <sub>2</sub> O
TTFA	0.1 mM	Ethanol

sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris/HCl pH 6.8, bromophenol blue and 1% DTT). The solvents DMSO and ethanol (Table 1) had no effect on  $^{35}S$ -methionine incorporation at the final concentrations (not higher than 1% v/v and 1.5% v/v respectively) obtained in experiments with water-insoluble inhibitors (data not shown). Half of each sample was separated in 12–20% SDS-polyacrylamide gradient gels.

**Estimation of  $^{35}S$ -incorporation into mitochondrial proteins.** Incorporation of  $^{35}S$ -methionine was estimated by the densitometry of autoradiographs using a Personal Densitometer, Molecular Dynamics, and ImageQuant (v 1.11) as a quantification computer program. All the values are expressed relative to the control value except for Fig. 7 where the absolute values of the detected signal were employed.

**Measurement of oxygen consumption.** Oxygen uptake of the isolated mitochondria was measured with a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) at 25°C in a total volume of 0.4 ml of the *in organello* synthesis medium. The effect of the different additions on oxygen uptake was calculated from the traces and plotted in panel B, Figs. 2, 3 and 4.

**Estimation of  $^{35}S$ -methionine uptake.** Mitochondria were incubated, according to the procedure for translation, with 200 µM of chloramphenicol to prevent mitochondrial translation. After incubation, mitochondria were centrifuged, washed twice with washing medium (0.4 M mannitol and 10 mM  $KH_2PO_4$ ) and re-suspended in 30 µl of



the same medium. Half of the volume was mixed with sample buffer at double solute concentration and analysed by gel electrophoresis and autoradiography. The other half was mixed with ReadySafe scintillation liquid (Beckman) and, following the manufacturer's instructions, estimation of the isotope was performed in a LS 6000IC Beckman scintillation counter. <sup>35</sup>S-methionine was obtained from Amersham. Chemicals for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. Other chemicals used were from Sigma or Boehringer-Mannheim.

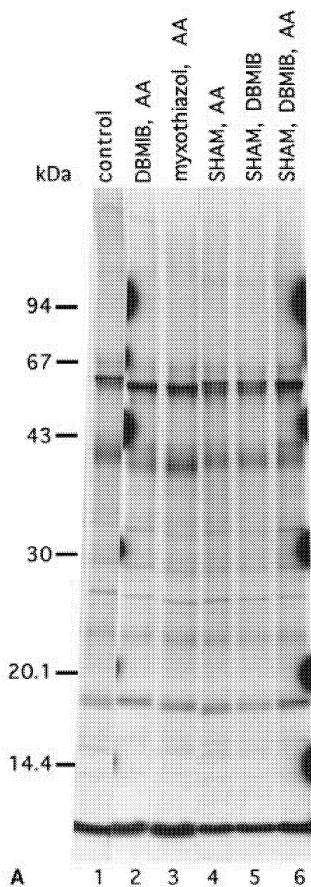
## Results

Figures 2–6, panel A, show autoradiographs of <sup>35</sup>S-methionine-labelled proteins separated in SDS-polyacrylamide gels after their synthesis de novo in intact mitochondria isolated from pea (*P. sativum* L.). Panels B show rates of oxygen consumption and/or total <sup>35</sup>S-methionine incorporation corresponding to the different additions shown in panels A.

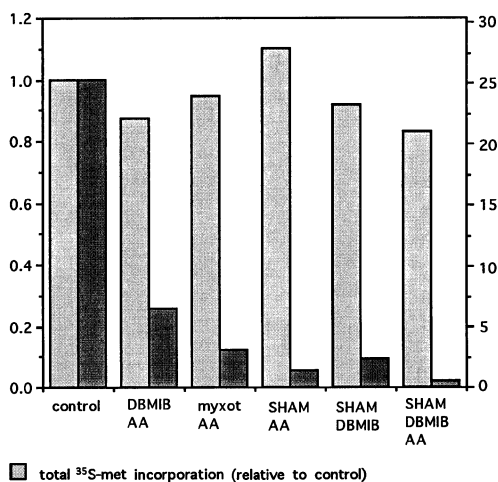
### Effects of reducing and oxidising conditions on mitochondrial protein synthesis

Figure 2 summarises the effect on <sup>35</sup>S-methionine incorporation into mitochondrial proteins obtained by inhibiting electron-transport at the beginning or the end of the respiratory chain (see Fig. 1). The polypeptide pattern observed in the control (Fig. 2A, lane 1) corresponds to mitochondrial translation products reported before, where the 55-kDa band is most likely the  $\alpha$  subunit of the F<sub>1</sub>-ATPase, the 39–40-kDa bands are the cytochrome *c* oxidase subunits I and II, and the 12-kDa band is subunit 9 of the ATPase (Lonsdale 1989). Under oxidising conditions created by the presence of the inhibitors rotenone, thenoyltrifluoroacetone (TTFA) and dicumarol, which cause inhibition of complexes donating electrons to the ubiquinone (UQ) pool (see Fig. 1), protein synthesis is almost completely inhibited (Fig. 2A, lane 2). On the other hand, salicylhydroxamic acid (SHAM) and potassium cyanide (KCN) inhibit electron transfer from the respiratory chain to O<sub>2</sub>, causing all the complexes of the respiratory chain to become reduced in the presence of oxidisable substrates (see Fig. 1). Under such reducing conditions mitochondrial protein synthesis is retained (Fig. 2A, lane 3). The observed differences in protein synthesis (lane 2 vs lane 3) are not correlated with respiratory activity, as measured by oxygen uptake, since both sets of inhibitors cause a dras-

**Fig. 2A, B** Effects of respiratory chain inhibitors on <sup>35</sup>S-methionine-labelling of proteins synthesised by isolated pea mitochondria. Malic acid and Na-pyruvate were added to all the reactions as respiratory substrates. Respiratory chain inhibitors were added as in Table 1. **A** autoradiograph of proteins separated by SDS-PAGE. **B** effect of inhibitors on total amounts of <sup>35</sup>S-methionine-incorporation into mitochondrial proteins and on oxygen consumption. The incorporation of <sup>35</sup>S-methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample



**A** 1 2 3 4 5 6



**B** total <sup>35</sup>S-met incorporation (relative to control)

oxygen uptake (nmol O<sub>2</sub> · mg protein<sup>-1</sup> · min<sup>-1</sup>)

tic reduction of oxygen consumption (Fig. 2B). In agreement with these results, and as previously described (Allen et al. 1995), inhibition of protein synthesis was also obtained in the presence of the oxidising agent potassium ferri-cyanide, while no inhibition occurred in the presence of the reducing agent DTT.

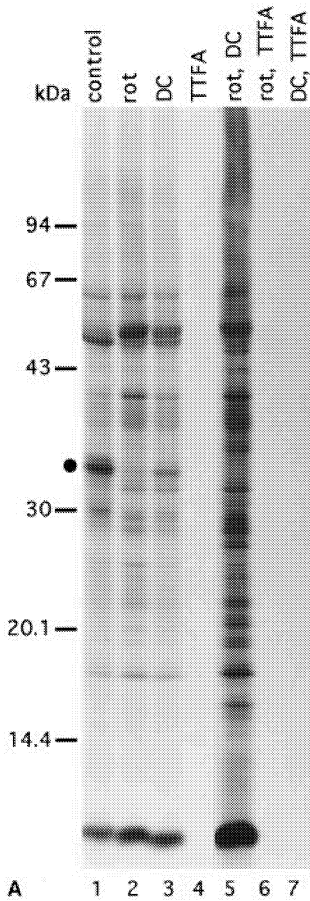
### Complexes III and IV

Figure 3 shows the oxygen uptake and polypeptide labelling obtained in the presence of different inhibitors that act specifically on complex III (Esposti et al. 1994). SHAM, an inhibitor of the alternative oxidase, was used together with the complex-III inhibitors in order to rule out the possibility of electron flow through the alternative oxidase. Antimycin A (Fig. 3A, lanes 2, 3, 4, and 6) interacts with the Q<sub>i</sub>-site of complex III (Fig. 1) and causes the reduction of cytochrome *b*. The inhibitors 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) (Fig. 3A, lanes 2, 5 and 6) and myxothiazol (Fig. 3A, lane 3), on the other hand, act specifically on the Q<sub>o</sub>-site of complex III (Fig. 1). Together with antimycin A, myxothiazol and DBMIB cause the oxidation of cytochrome *b*. No major differences were seen in the pattern of <sup>35</sup>S-methionine incorporation between samples incubated in the presence of Q<sub>i</sub>- or Q<sub>o</sub>-site inhibitors. Therefore, it is unlikely that there is a regulatory component for the redox control of protein translation within complex III.

Inhibition of complex III (Fig. 3A) causes the oxidation of complex IV while addition of SHAM and KCN (Fig. 2A, lane 3) cause its reduction; no difference in protein patterns, as compared to the control, was observed in these two cases. It is unlikely therefore that the observed effects on protein synthesis seen in Fig. 2 (lane 2 compared with lane 3) results from the action of any redox sensor in complex IV.

This conclusion is strengthened by further experiments. As stated earlier, oxidation of the ubiquinone pool and subsequent electron carriers, caused by the presence of rotenone, TTFA and dicumarol, resulted in the inhibition of protein synthesis. This effect could not be reversed by the addition of up to 1 mM of duroquinol (data not shown), which is able to feed electrons to the respiratory chain at the ubiquinone pool level, thus reducing complexes III and IV.

**Fig. 3A, B** Effects of complex-III inhibitors on <sup>35</sup>S-methionine-labelling of proteins synthesised by isolated pea mitochondria. Malic acid and Na-pyruvate were added to all the reactions as respiratory substrates. The alternative oxidase inhibitor SHAM was included in some of the reactions. Respiratory chain inhibitors were added as in Table 1. **A** autoradiograph of proteins separated by SDS-PAGE. **B** effect of inhibitors on total amounts of <sup>35</sup>S-methionine-incorporation into mitochondrial proteins and on oxygen consumption. The incorporation of <sup>35</sup>S-methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample

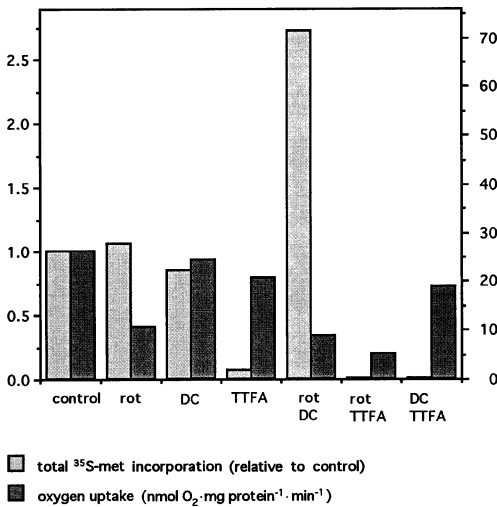


Complexes donating electrons to the ubiquinone pool

To distinguish between the respiratory chain components located prior to the UQ-pool, different combinations of inhibitors for these complexes were employed (Fig. 4). The presence of TTFA in these experiments showed that the activity of complex II is necessary to support the incorporation of <sup>35</sup>S-methionine into mitochondrial proteins (Fig. 4A, lanes 4, 6 and 7).

In contrast with this observation, inhibition of complex I (addition of rotenone, lane 2) or the internal and external NADH dehydrogenases (addition of dicumarol, lane 3) does not affect protein synthesis. However, inhibition of all NADH dehydrogenases (rotenone plus dicumarol, lane 5) has a stimulatory effect on mitochondrial protein synthesis. Our results suggest that the activity of complex II is required for protein synthesis, since the addition of TTFA always causes an inhibition of protein synthesis (Fig. 4, lanes 4, 6 and 7; Fig. 2, lane 2). Inhibition of all the NADH-dehydrogenases, on the other hand, results in higher amounts of <sup>35</sup>S-methionine incorporation, but only when complex II is still active (Fig. 4A, lane 5 vs Fig. 2, lane 2). Besides these general effects on protein synthesis, alterations in the labelling intensity of specific protein bands were sometimes found after the addition of a respiratory inhibitor (Fig. 4A). These alterations in protein patterns were, however, not consistent.

As described earlier (Fig. 2B), no correlation between oxygen uptake and the incorporation of <sup>35</sup>S-methionine into mitochondrial proteins was observed (Fig. 4B). Oxygen consumption is still high in the presence of TTFA (since the activity of complex I maintains respiratory activity) while protein synthesis is inhibited. By contrast, the addition of dicumarol together with rotenone reduces oxygen consumption to half of the control level, while protein synthesis is strongly induced.



Inactivation of the TCA cycle

It was important to determine whether the effect of TTFA on mitochondrial protein synthesis was a result of a direct inhibition of electron-transport within the complex or of an indirect inhibition of the TCA cycle.

Fig. 4A, B Effects of respiratory chain inhibitors on <sup>35</sup>S-methionine-labelling of proteins synthesised by isolated pea mitochondria. These inhibitors prevent electron donation to the UQ-pool. Malic acid and Na-pyruvate were added to all the reactions as respiratory substrates. Respiratory chain inhibitors were added as in Table 1. A autoradiograph of proteins separated by SDS-PAGE. The dot represents a protein band with alterations in intensity between experiments. B effect of inhibitors on total amounts of <sup>35</sup>S-methionine-incorporation into mitochondrial proteins and on oxygen consumption. The incorporation of <sup>35</sup>S-methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample

### Incubation at 25°C

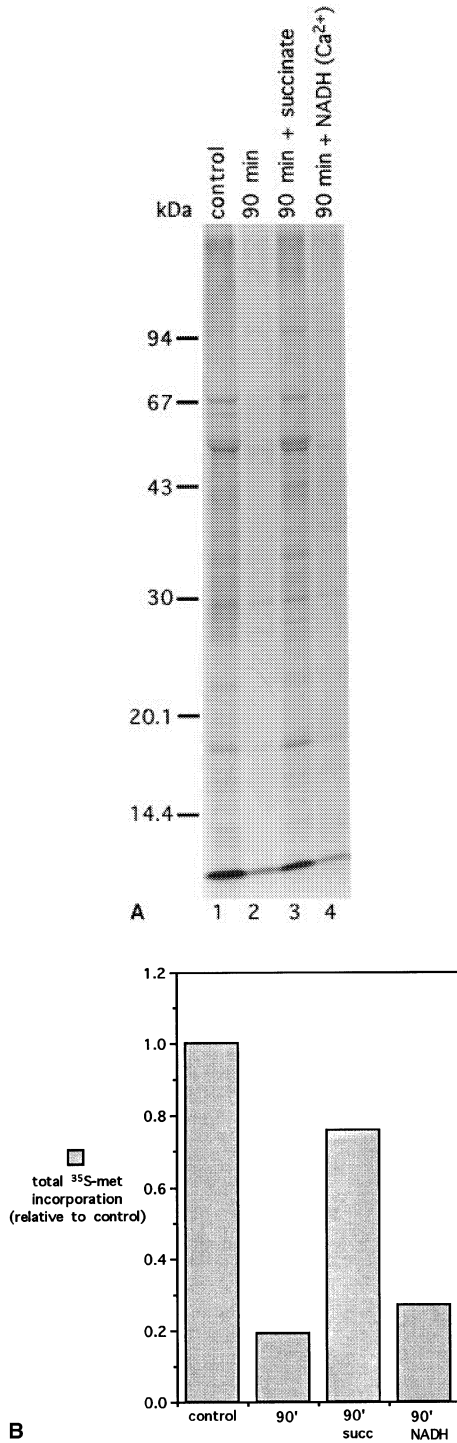
In further experiments, isolated mitochondria were allowed to consume endogenous substrates by pre-incubation in synthesis medium, at 25°C, on a shaker for 90 min. Labelled translation was initiated by the addition of  $^{35}\text{S}$ -methionine. The polypeptide pattern obtained after pre-incubation (Fig. 5A, lane 2) showed a substantial decrease in labelling compared to that of the control (lane 1). Partial restoration of the protein pattern was attained by the addition of succinate before the labelled translation was initiated (lane 3).

Figure 5 also shows the results of the addition of NADH with  $\text{Ca}^{2+}$  (addition of  $\text{Ca}^{2+}$  is necessary for activation of the external NADH dehydrogenase) instead of succinate (Fig. 5A, lane 4). In contrast to succinate, this respiratory substrate was unable to restore the original translation pattern to any degree. Oxidation of succinate ( $25.7 \text{ nmol O}_2 \cdot \text{mg of protein}^{-1} \cdot \text{min}^{-1}$ ) and NADH ( $18.7 \text{ nmol O}_2 \cdot \text{mg of protein}^{-1} \cdot \text{min}^{-1}$ ) was measured in the oxygen electrode with the mitochondria incubated as described above, in order to show that the mitochondria were still able to oxidise these substrates after the pre-incubation period.

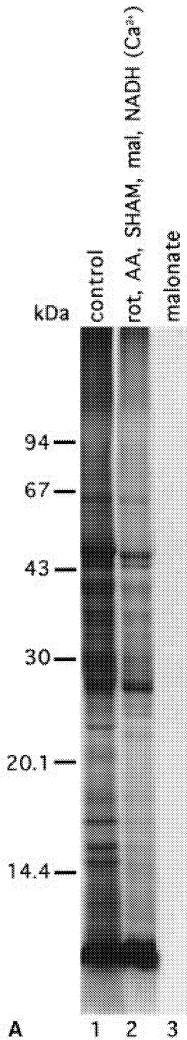
These results further point to a specific role of complex II in the regulation of mitochondrial protein synthesis.

### Addition of malonate

Malonate, another inhibitor of complex II, has the same effect on protein synthesis as TTFA (Fig. 6A, lane 3), although it affects electron flow through the complex in a different way, namely by blocking the active site of the large subunit of succinate dehydrogenase (Wilson et al. 1996). TTFA, on the other hand, interacts with the quinone-binding site of the complex (Ramsay et al. 1981; Chauveau and Roussaux 1996). The presence of malonate causes the oxidation of the complex, and at the same time inhibits the TCA cycle. Reduction of complex II, in the presence of malonate, was achieved by the reverse-flow of electrons from the ubiquinone pool. Electrons were fed through the external NADH dehydrogenase by the addition of NADH and  $\text{Ca}^{2+}$ . If at the same time electron-transport through complex I and III (addition of rotenone, antimycin A) and the alternative oxidase (presence of SHAM) is inhibited, a flow of electrons from the reduced ubiqui-



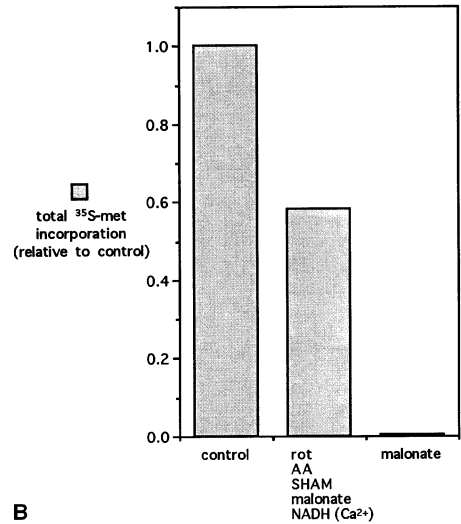
**Fig. 5A, B** Restoration, by addition of succinate, of  $^{35}\text{S}$ -methionine-incorporation into mitochondrial proteins after depletion of endogenous substrates by pre-incubation for 90 min. Synthesis medium was used as a pre-incubation medium. **A** autoradiograph of proteins separated by SDS-PAGE. *Lane 1* control; *lane 2* incubation for 90 min; *lane 3* addition of succinate after incubation for 90 min; *lane 4* addition of NADH and  $\text{Ca}^{2+}$  after incubation for 90 min. Final concentrations of additions as in Table 1. **B** effects on total amounts of  $^{35}\text{S}$ -methionine-incorporation into mitochondrial proteins. The incorporation of  $^{35}\text{S}$ -methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample.



none pool to complex II occurs. With this procedure a partial recovery of the initial rate of labelling was observed (Fig. 6A, lane 2).

#### Influence of ATP concentration

In addition to the investigation of the effect of electron-transport inhibitors, the effect of ATP concentration was studied, in order to rule out the possibility that the results obtained (Fig. 4) merely reflect changes in the rate of ATP production produced by the different inhibitors. ATP titrations were carried out in the absence and presence of 4 mM of ADP (Fig. 7A and B).



**Fig. 6A, B** Restoration, by re-reduction of complex II, of the <sup>35</sup>S-labelled mitochondrial protein pattern obtained in the presence of malonate. Final concentrations of additions as in Table 1. **A** autoradiograph of proteins separated by SDS-PAGE. **B** effects on total amounts of <sup>35</sup>S-methionine incorporation into mitochondrial proteins. The incorporation of <sup>35</sup>S-methionine is presented in relative units where a value of 1 corresponds to the incorporation of the control sample

In both cases, absence and presence of ADP, the addition of TTFA caused an inhibition of mitochondrial protein synthesis at all the ATP concentrations employed. The higher values of incorporation at low ATP concentrations, in the presence of ADP (Fig. 7B), were mainly a result of the higher incorporation of label into a single protein, the proposed *atp9* gene product (data not shown; for position of *atp9* see Fig. 2A).

In contrast to TTFA, addition of the NADH-dehydrogenase inhibitors, rotenone and dicumarol, did not change <sup>35</sup>S-methionine incorporation as compared to the control. At the highest ATP concentrations (4 mM) the addition of dicumarol and rotenone increase protein synthesis relative to that of the control, possibly because of high concentrations of ATP in the absence of respiratory inhibitors. Previous work (Lind et al. 1991) has shown that the incorporation of <sup>35</sup>S-methionine was inhibited by the external addition of ATP.

On the basis of these observations (Fig. 7), we conclude that the observed effect of TTFA on mitochondrial protein synthesis is not a result of changes in ATP concentration. While the highest values of incorporation for the control were seen at around 3 mM of ATP (Fig. 7A), not even 4 mM of ATP was enough to support protein synthesis in the presence of TTFA.

As mentioned before, respiratory activity and the incorporation of label showed no correlation, strengthening the conclusion that the observed effects are not the result of

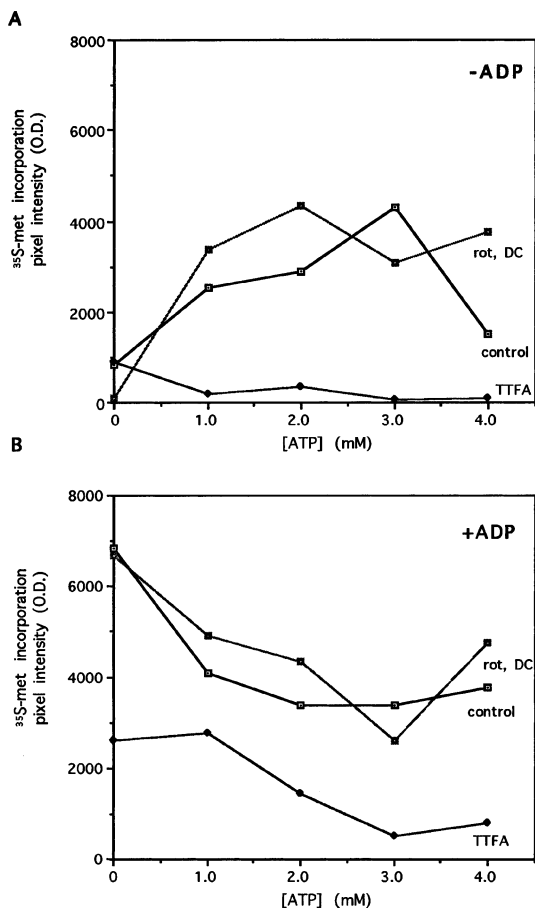


Fig. 7. **A, B** Effect of different concentrations of ATP on the incorporation of labelled methionine into mitochondrial proteins. Malic acid and Na-pyruvate were added to all the reactions as respiratory substrates. **A** in the absence of ADP. **B** in the presence of 4 mM of external ADP

changes in ATP concentration caused by the presence of the different respiratory inhibitors.

#### Effect of TTFA on amino-acid uptake

It could be argued that the effect of TTFA on mitochondrial protein synthesis is a result of changes in the uptake of  $^{35}\text{S}$ -methionine caused by the presence of this respiratory inhibitor. In order to investigate this possibility, mitochondria were incubated in medium containing chloramphenicol, an inhibitor of mitochondrial protein synthesis, in the presence and absence of TTFA. The uptake of labelled methionine was estimated by scintillation counting of the washed and re-suspended mitochondrial pellet. No significant differences ( $P=0.8773$ , 95% confidence level)

in the uptake of labelled methionine were observed between samples with ( $155\ 440 \pm 12\ 538$  cpm) and without TTFA ( $151\ 213 \pm 22\ 422$  cpm).

## Discussion

Oxidising conditions of the mitochondrial electron-transport chain created by the inhibition of the electron-donating complexes (Fig. 2) inhibit mitochondrial protein synthesis. In previous work (Allen et al. 1995) addition of the oxidising agent potassium ferricyanide was shown to have a similar effect, suggesting that mitochondrial protein synthesis is controlled by oxidation-reduction reactions. These results favour the hypothesis that mitochondrial translation is inhibited when some component of the respiratory chain, which therefore acts as a redox sensor (Allen 1993a), becomes oxidised. In agreement with this, previous work (Danon et al. 1994) has shown that reducing conditions promote translation in isolated chloroplasts. Pearson et al. (1993) and Wilson et al. (1996), on the other hand, showed that UTP incorporation into chloroplast and mitochondrial RNA, in isolated plant organelles, was inhibited when specific components of the electron-transport chain were reduced. These examples of the redox regulation of organelle gene expression in plants might indicate differential regulation depending on the level of gene expression.

It was found that the activity of complex II is necessary for mitochondrial protein synthesis (Fig. 4). Inhibition of complex I and at the same time of alternative NADH dehydrogenases, on the other hand, promotes protein synthesis. The increase in the rate of incorporation of labelled methionine into mitochondrial proteins upon inhibition of the NADH dehydrogenases could result from the production of inhibitory concentrations of ATP when these complexes are active (Fig. 7). However, since rotenone and dicumarol also increase protein synthesis at low ATP concentrations, these results could indicate the presence of at least two regulatory points. Low activity of the NADH dehydrogenases together with an active complex II promotes protein synthesis. It could be argued that this effect reflects a physiological state where the production of reducing power by the TCA cycle exceeds the limits of its consumption by the respiratory chain. Under such conditions there will be a demand of new components of the electron-transport chain, and thus protein synthesis needs to be up-regulated. Under these same conditions succinate dehydrogenase activity will be high. Alterations in the redox state of the succinate:ubiquinone oxidoreductase might therefore be a key regulatory point for protein synthesis in mitochondria. Care has to be taken, though, in interpreting these results since all experiments were carried out with isolated mitochondria, separated from the rest of the cell.

While succinate dehydrogenase creates a direct connection between the TCA cycle and the respiratory chain, all other TCA-cycle enzymes are located in the matrix. Succinate dehydrogenase activity depends on succinate pro-



duction and therefore TCA-cycle activity. The activity of the TCA cycle depends on the oxidation of NADH and FADH<sub>2</sub> by the respiratory chain. Succinate dehydrogenase is also one of the most active enzymes in isolated mitochondria, showing very complex kinetics, suggesting a possible regulatory activity. However, no evidence for a regulatory role of succinate dehydrogenase in the TCA cycle has been found (Wiskich and Dry 1985).

The absence of incorporation of <sup>35</sup>S-methionine into newly synthesised polypeptides after TTFA treatment could be caused either by inhibition of the TCA cycle or by the blocking of electron-transport through complex II. In cases where mitochondria were starved of endogenous respiratory substrates before labelled translation was initiated (Fig. 5) the TCA cycle becomes inhibited, since it is depleted of substrates (NAD<sup>+</sup>). In these cases, partial recovery of translation with the addition of succinate prior to the initiation of labelled translation indicates that the activity of the TCA cycle is not correlated with the regulation of mitochondrial protein synthesis. This conclusion is strengthened by experiments where, in the presence of malonate, protein synthesis was supported by an excess of electrons in the ubiquinone pool, having complex II as the only acceptor (Fig. 6, lane 2). This result suggests that the electron flow within the complex is sufficient to support mitochondrial protein synthesis, at least partially, in spite of the inhibition of the TCA cycle. The lack of a full recovery of protein synthesis could reflect the fact that is difficult to obtain full reduction of complex II through flow-back from the UQ-pool. We suggest that the effect of TTFA on protein synthesis is most likely the result of a change in the redox state of complex II or of some component closely associated with this complex.

Oxidation of succinate to fumarate causes reduction of the FAD bound near to the active site of the large subunit (Fp) of complex II (Cramer and Knaff 1991; Igamberdiev and Falaleeva 1994). Electron transfer from FAD to the ubiquinone pool apparently involves two of the three iron-sulphur clusters (S-1 and S-3). The role of the other centre (S-2) is not clear, but a complex interaction with S-1 has been found. Although the different inhibitors (malonate and TTFA) for complex II used in our investigation have different sites of action, they both inhibit mitochondrial protein synthesis. Malonate competes with succinate for the active site on Fp, causing oxidation of the whole complex. TTFA, on the other hand, blocks the electron flow from S-3 to the ubiquinone pool, perhaps at the binding site of the quinone pair (Ramsay et al. 1981), which would lead to reduction of the complex. Recent work (Chauveau and Roussaux 1996) has shown two possible sites of inhibition by TTFA and corroborated its direct interaction with S-3, which would imply a partial reduction of complex II in the presence of TTFA. However, since electron flow within the complex has not been well established, we cannot determine how the redox state of the different components is affected by TTFA, and thus we cannot attribute the function of a redox sensor to any specific component of the complex. Since the mid-point redox potentials ( $E_{m7}$ ) for the different components are known (Cramer and Knaff 1991), it

should be possible to identify the redox sensor within the complex by redox titration (Allen and Holmes 1986).

We conclude that our results are consistent with the hypothesis that the mitochondrial genetic system permits direct redox regulatory control of gene expression by respiratory electron-transport (Allen 1993b). The specific requirement for the activity of complex II suggests that a redox sensor involved in such a regulation is contained within, or close to, complex II. Further work is required in order to establish the mechanisms which couple electron-transport to protein synthesis in mitochondria, and to determine whether this is the only level of gene expression at which such control is exerted.

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*II*



# MITOCHONDRIAL GENE EXPRESSION IN PEA IS REGULATED BY THE ACTIVITY OF RESPIRATORY COMPLEX II

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## Introduction

Protein synthesis in isolated pea mitochondria has been shown to be dependent on the activity of respiratory complex II (Escobar Galvis *et al.*, 1998). Inhibition of electron transport through this complex causes complete inhibition of <sup>35</sup>S-methionine incorporation into mitochondrial proteins. The evidence presented points to the redox state of complex II as a regulatory factor in mitochondrial gene expression, although the mechanism of regulation and the level at which control is exerted have not yet been clarified. Here we demonstrate partial inhibition of incorporation of <sup>32</sup>P-labelled UTP, in isolated and permeabilised mitochondria, by thenoyltrifluoroacetone (TTFA), a specific inhibitor of complex II. Our data suggest that although some regulation of transcription seems to occur, the redox state of complex II regulates mitochondrial gene expression mainly at the translational level. We further show that protein synthesis is accompanied by phosphorylation of a 13 kDa protein, not previously detected in phosphorylation assays carried out in pea mitochondria (Håkansson and Allen 1995). Incorporation of label from [ $\gamma$ -<sup>32</sup>P]ATP into the 13 kDa protein is also inhibited by TTFA. These preliminary observations raise the possibility that protein phosphorylation could play a role in signal transduction between complex II and the mitochondrial protein synthesis apparatus. Our observations support the hypothesis that the mitochondrial genetic system permits direct regulatory control of gene expression upon changes in the redox state of electron carriers of the respiratory chain (Allen 1993).

## Materials and Methods

Mitochondria were isolated from young pea leaves (11-12 days old, grown at 20°C with a 12-h day) according to Boutry *et al.* (1984) with modifications according to Håkansson *et al.* (1988).

*In organello* translation was carried out according to Escobar Galvis *et al.* (1998). Incorporation of labelling was estimated by densitometry of autoradiographs using a Personal Densitometer, Molecular Dynamics and ImageQuant (v 1.11).

RNA synthesis assays were carried out according to Muise and Hauswirth (1992). Total counts of incorporated isotope were acid precipitated and counted by liquid scintillation in a LS 6000IC Beckman scintillation counter.

After mitochondrial translations were carried out the pelleted mitochondria were resuspended in assay mixture (Petit *et al.* 1990). Phosphorylation assays were carried out according to Håkansson and Allen (1995). Proteins were separated by SDS-PAGE and phosphoproteins were detected by autoradiography.

## Results and Discussion

### Translation and RNA synthesis assays

Figure 1 shows the effects of addition of TTFA and actinomycin D on incorporation of  $^{35}\text{S}$ -methionine and  $^{32}\text{P}$ -UTP into mitochondrial proteins and RNA, respectively. Addition of actinomycin D, a potent inhibitor of the mitochondrial transcription, caused a decrease in  $^{35}\text{S}$ -methionine incorporation of about 30%, suggesting the presence of a population of stable mRNAs that are translated during the *in organello* assay. It was also found that addition of TTFA decreased the incorporation of  $^{32}\text{P}$ -UTP into TCA precipitated RNA by about 40%. Although inhibition of incorporation of labelling was observed, this inhibition does not explain the complete absence of  $^{35}\text{S}$ -methionine incorporation into mitochondrial proteins in the presence of TTFA.

Our results suggest that the effect of TTFA on protein synthesis is exerted mainly at the translational level, having a limited effect on mitochondrial transcription. Thus, the activity of respiratory complex II seems to regulate mitochondrial translation while mitochondrial transcription might involve additional regulatory points. Addition of rotenone (a complex I inhibitor) and dicumarol (an inhibitor of NADH dehydrogenases) had no significant effect on the incorporation of  $^{32}\text{P}$ -UTP (data not shown). Since no other inhibitors were tested in these experiments, a role for other electron carriers in regulation of mitochondrial transcription cannot be excluded. Wilson *et al.* (1996) suggested complex III to be involved in redox regulation of RNA synthesis in potato mitochondria.

### Protein synthesis and phosphorylation

In fig. 2, the phosphorylation of a 13 kDa protein (indicated by a dot) is seen to be correlated with protein synthesis. Incubation of mitochondria, which had previously been carried through a protein synthesis assay, with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , caused the phosphorylation of the additional 13 kDa band (lane 2), as compared to the control (lane 1). Presence of TTFA (lane 3) caused inhibition of the phosphorylation of this band. Effects of TTFA on phosphorylation of minor bands at about 25, 60 and 70 kDa may also have occurred, but labelling of these

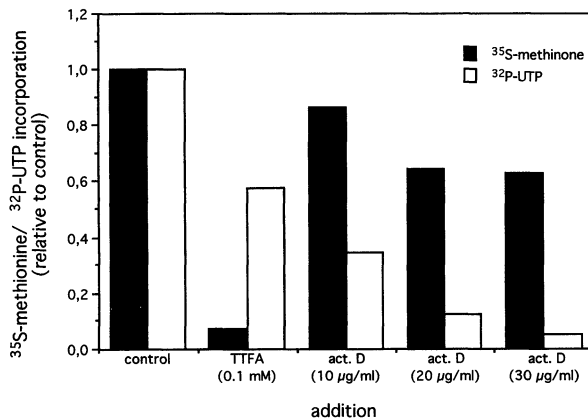


Fig. 1. Effect of TTFA and actinomycin D additions on incorporation of  $^{35}\text{S}$ -methionine and  $^{32}\text{P}$ -UTP into mitochondrial proteins and RNA respectively. Incorporation of the isotopes is presented in relative units where a value of one corresponds to the incorporation of the control sample.

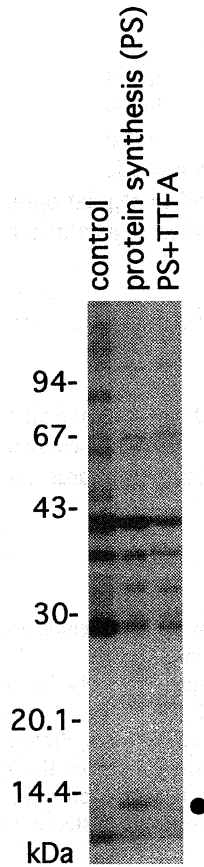


Fig. 2. Effect of protein synthesis on mitochondrial protein phosphorylation. Autoradiograph of  $^{32}\text{P}$ -labelled proteins separated by SDS-PAGE. The dot indicates a labelled 13 kDa protein, phosphorylation of which is clearly associated with protein synthesis. TTFA was added to give a final concentration of 0.1 mM.

bands is not correlated with protein synthesis since they are also observed in the control (lane 1). As a control, mitochondria were incubated in washing medium (0.4 M mannitol, 10 mM  $\text{KH}_2\text{PO}_4$ , pH 7.2) for the same length of time as in the translation assay, and the phosphorylation assay, was then carried out as for the rest of the samples. In this case labelling of the 13 kDa protein was not observed (data not shown).

Since the phosphorylation of the 13 kDa protein occurred only after protein synthesis had taken place, we suggest that its phosphorylation is associated with mitochondrial translation. One can not, however, rule out the possibility that the mitochondria became depleted of some factor during protein synthesis, a factor which then induces phosphorylation of the 13 kDa band. On the other hand, our hypothesis is supported by the observation that TTFA, a complex II inhibitor, inhibits mitochondrial protein synthesis (Escobar Galvis *et al.* 1998) as well as the phosphorylation of the 13 kDa protein. Based on these preliminary findings we propose that phosphorylation of the 13 kDa protein might be part of a signal transduction pathway that controls mitochondrial gene expression in response to changes in the redox state of complex II (Escobar Galvis *et al.* 1998). This hypothesis is presently under investigation in our laboratory.

## Conclusions

Regulation of gene expression in isolated pea mitochondria is controlled by the redox poise of complex II, at least at the translational level. Mitochondrial transcription seems to be under control of additional components that have not yet been identified. Further work, including use of inhibitors of other electron carriers, might help to elucidate if other components of the respiratory chain are involved in regulation of mitochondrial transcription. We further propose that protein phosphorylation is part of a redox regulatory system that controls mitochondrial gene expression.

## Acknowledgements

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***III***



## Cloning and characterisation of a pea mitochondrial NDPK

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**Abstract** — Here we report the cloning of a cDNA encoding the first nucleoside diphosphate kinase (NDPK) isolated from plant mitochondria. Amplification of a 317 nt product was performed by PCR, using oligonucleotide primers based on partial amino acid sequences of the pea mitochondria NDPK and other NDPK isoforms. By screening of a pea cDNA library with this PCR product, a full length clone was obtained. Northern analysis revealed the presence of a 1.1 kb single transcript, with high expression in young leaves and reproductive tissues. The clone encodes a precursor protein of 232 amino acids (26 kDa), including an *N*-terminal extension of 80 amino acids (9 kDa). Analysis of the deduced amino acid sequence confirmed its identity with the sequences obtained from the purified mature pea mitochondrial NDPK. In vitro import experiments carried out in isolated pea mitochondria showed targeting and processing of the 27 kDa precursor into a 16.5 kDa mature form. Phylogenetic analysis of some vertebrate and plant isoforms of NDPK showed that the pea mtNDPK groups together with the NDPK3 isoform from *A. thaliana* and the chloroplastic NDPK III from spinach. We suggest that it is possible to design a novel classification of the different NDPK isoforms according to their subcellular localisation and origin. © 1999 Société française de biochimie et biologie moléculaire/Éditions scientifiques et médicales Elsevier SAS

*Pisum sativum* / mitochondria / nucleoside diphosphate kinase / cDNA / protein import

### 1. Introduction

The nucleoside diphosphate kinases (NDPKs) are enzymes whose major role is to maintain the balance between adenine and non-adenine triphosphates. NDPKs catalyse the transfer of phosphate from  $N_1$ TP to  $N_2$ DP to form  $N_2$ TP, via a NDPK-phospho-histidine intermediate [2]. Besides this housekeeping function, several examples of the role of NDPK in cell signalling have been described [3, 4]. Some of the animal isoforms play key roles in processes such as cell proliferation [5], cell differentiation [6] and transcriptional regulation [7]. In contrast, much less is known about the possible role of plant NDPKs in cell signalling. The only experimental evidence of such a role has been found in pea, where NDPK I, a cytosolic isoform, becomes phosphorylated upon red-light irradiation, in a process which is believed to involve phytochrome B [8] and in *A. thaliana* where an NDPK isoform is thought to be involved in UV-B light signalling [9].

NDPKs are ubiquitous enzymes whose activity has been detected in various subcellular compartments. In

*Arabidopsis* [10], pea [11] and spinach [12], both chloroplastic and cytoplasmic NDPKs have been identified. Although NDPK activity has been detected in mitochondria isolated from several types of organisms and tissues [2, 13] (and references therein) only two mitochondrial isoforms have been isolated. One from pigeon, located in the matrix [14] and one from pea, with a suggested intermembrane localisation [1]. In addition, two cDNA clones, *nm23-H4* from human [15] and *guk* from *Dictyostelium discoideum* [16], with putative mitochondrial targeting peptides, have been isolated.

Mitochondrial NDPK activity varies between different tissues and organisms, and the roles of mitochondrial isoforms are under discussion. A reasonable role for a matrix located NDPK in animals could be to catalyse the transfer of the phosphoryl group from GTP, synthesised by the succinyl-CoA synthetase in the TCA cycle, to form ATP. However, in heart mitochondria isolated from pigeon, where high activity of the TCA cycle has been detected, NDPK activity was found to be low and mainly located outside the inner membrane [17]. In plants, ATP is synthesised directly by the succinyl-CoA synthetase [18], questioning the need for a matrix mitochondrial isoform with this particular function. NDPK activity in the mitochondrial intermembrane space has been detected in various organisms and a role in the generation of NTPs for the cytosol using the mitochondrially produced ATP was proposed in early experiments [13]. An intermembrane space located NDPK could thus play a role in communi-

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**Abbreviations:** NDPK, nucleoside diphosphate kinase; PCR, polymerase chain reaction; aa, amino acids; MPP, mitochondrial processing peptidase; IMP, inner membrane peptidase; TPP, thylakoid processing peptidase; IMS, intermembrane space.

cation between mitochondria and cytosol rather than in maintaining the balance of triphosphates inside the mitochondria.

Recently, a mitochondrial NDPK isoform from pea leaves was isolated and purified [1]. Its *N*-terminal sequence showed high similarity to the human cytosolic isoforms Nm23-H1 and Nm23-H2, that have been shown to be involved in intracellular signalling [3, 7]. The similarity in sequence and in biochemical features, such as serine auto-phosphorylation [1, 3], between the pea mitochondrial and human isoforms, raises the question whether this intermembrane space protein might play a role in signalling between subcellular compartments. Determination of the full sequence of the pea mitochondrial NDPK is the first step in its complete characterisation. In this paper we report cDNA cloning of the pea mitochondrial isoform, import into mitochondria and differential tissue and developmental expression of the gene.

## 2. Materials and methods

### 2.1. Plant material

Peas (*Pisum sativum*, L. cv Oregon sugar pod) were grown on vermiculite in a growing chamber at 20 °C with a 12 h light period (25  $\mu\text{E m}^{-2} \text{s}^{-1}$ ). Flowers, pods and senescent leaves were collected from plants growing on soil under greenhouse conditions (20 °C, 12 h day).

### 2.2. Polymerase chain reaction

The following degenerate oligonucleotides were used as primers for polymerase chain reaction (PCR). The forward primer 5'-GAGCTYGAGCGTACYTTYATYGC-3' (figure 1) corresponds to the protein sequence ELERTFIA obtained by *N*-terminal sequencing of a NDPK purified from pea leaf mitochondria [19]. The reverse primer 5'-CCTGGIACYATCCGTGGIATCCGTGGIGAYTT (figure 1) corresponds to a conserved sequence of several plant NDPKs. First-strand cDNA was synthesised by reverse transcription from total RNA isolated from 7-day-old pea leaves. The conditions used for PCR were as follows: 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 °C), 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 200  $\mu\text{M}$  of each of dATP, dCTP, dGTP and dTTP, 0.25  $\mu\text{g}$  of each primer, 2  $\mu\text{L}$  of cDNA (corresponding to 0.4  $\mu\text{g}$  of total RNA) and 2.5 units of Taq DNA polymerase (Promega) in a final volume of 100  $\mu\text{L}$ . A 317 bp PCR product was obtained after amplification (25 cycles, 1 min 95 °C, 1.5 min 54 °C and 1 min 72 °C).

### 2.3. cDNA library screening and sequencing

Total RNA was isolated from 7-day-old pea leaves according to Hughes et al. [20]. Purification of mRNA was

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1  MASHLCKXSASRAARSLLSASFHSQGRAVAAAAAASIRKV
41  PVFAPNRYRRTGSGNGPSSWIAGALALPAAAYMLQDQEVHA

```

**Figure 1.** Deduced amino acid sequence of the targeting sequence of the pea mitochondrial NDPK.

performed using Dynabeads (Dyna) according to the manufacturer's instructions. A cDNA library was constructed using a HybriZAP-2.1 two hybrid cDNA synthesis kit (Stratagene) following the manufacturer's instructions.

The PCR product was labelled and used to screen a HybriZAP-2.1 pea cDNA library. Approximately  $6 \times 10^5$  plaque-forming units were screened. Sequencing was performed on a ABI Prism 310 Genetic Analyser (Perkin-Elmer) using AmpliTaq DNA polymerase, FS (Perkin-Elmer) according to the manufacturer's instructions.

### 2.4. In vitro translation and import

A *Sall* fragment containing 12 bp upstream of the start codon and the entire coding region of the positive clone was subcloned into BlueScript (Stratagene) under the control of the T7 promoter. In vitro translation was carried out using a TNT Coupled Reticulocyte Lysate System (Promega), in the presence of [<sup>35</sup>S]methionine according to the manufacturer's instructions. Import assays were carried out as described earlier [21], using 200  $\mu\text{g}$  of intact mitochondria per assay. In organello phosphorylation assays were carried out according to Struglics and Håkansson [1, 19]. Mitochondria were isolated from 12–14-day-old pea leaves according to Boutry et al. [22]. Proteins were separated by SDS-PAGE (12–18% T gradient gels, C = 2.67%) according to Laemmli [23]. The [<sup>35</sup>S]methionine labelled proteins were visualised by autoradiography.

### 2.5. Computer-generated analysis

Sequences were analysed using the ClustalW program [24] and the phylogenetic tree was constructed by the PAUP 4.0B2 for program Macintosh [25].

### 2.6. RNA isolation and hybridisation

Total RNA was isolated from pea leaves (5–21 days old), flowers, pods and roots using the method of Hughes et al. [20]. RNA was separated in a 1% denaturing agarose gel using standard methods [26] and blotted onto Biodyne A membranes (Pall). Prehybridisation and hybridisation were carried out at 52 °C in 50% formamide, 3  $\times$  SSC (1  $\times$  SSC is 0.3 M NaCl and 0.03 M Na-citrate), 5  $\times$  Denhardt's solution (1  $\times$  Denhardt's is 0.02% polyvinylpyrrolidone, 0.02% Ficoll 400, 0.02% bovine serum

albumin), 0.08% SDS and 100  $\mu\text{g mL}^{-1}$  denatured salmon sperm DNA. High stringency washes were performed at 68 °C with 0.1  $\times$  SSC and 0.1% SDS. DNA from the positive cDNA clone was labelled using oligonucleotide labelling kit (Pharmacia) in the presence of [ $^{32}\text{P}$ ]dCTP and used as a probe.

### 3. Results

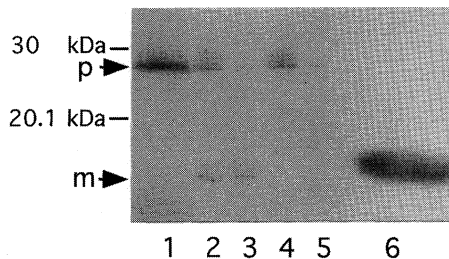
#### 3.1. Isolation of the mtNDPK cDNA from pea

In order to identify the cDNA coding for the pea mitochondrial NDPK, pea leaf cDNA was amplified using degenerate primers corresponding to the *N*-terminal sequence, as obtained by Struglics and Håkansson [19], and a conserved region from compiled sequences of NDPKs. A single 317 bp PCR product was obtained and sequenced. Using the labelled PCR product as probe, a full length positive clone was obtained after secondary screening of a pea HybriZAP-2.1 cDNA library. Sequence analysis confirmed its correspondence with the isolated protein [1]. The deduced amino acid (aa) sequence showed high similarity with other NDPKs found in databases (Swissprot, TREMBL, GenBank) by BLAST searches [27].

#### 3.2. Sequence analysis

The longest ORF of 232 aa starts at position 56 of the full-length cDNA (data not shown, see GenBank accession no. AF191098 for sequence). The 3' end contains a predicted poly-A addition site at position 818. The TGA stop codon is followed by a 271 nt long untranslated region and a short poly-A tail of 18 adenylate residues.

The deduced amino acid sequence corresponding to the mature protein was identical to the partial sequences obtained from the isolated pea mtNDPK [1, 19]. It also contains an *N*-terminal extension of 80 aa with respect to the start of the mature protein as sequenced by Struglics and Håkansson [19] (figure 1). The overall structure of the targeting sequence is predicted to be helix-random coiled-helix [28]. The first part of the first helix (17 aa), is amphiphilic, a structure that has been shown to be important for mitochondrial targeting [29]. The second helix forms a hydrophobic structure that resembles an intramitochondrial sorting signal according to the postulated criteria in the algorithm used by the PSORT program [30]. A clear consensus feature for the mitochondrial processing peptidase (MPP) has been shown to be the presence of an arginine at the -2 or -8 position relative to the cleavage site [31]. No arginine can be found at the -2 or -8 position of the *N*-terminus of the mature protein, moreover, the closest arginine is located at position -32. Processing at this position would result in a form intermediate in size between the precursor and the mature



**Figure 2.** In vitro import and in organello phosphorylation of isolated pea leaf mitochondria. Lane 1, in vitro translation product of the pea mtNDPK. Lanes 2, 3, in vitro import of the pea mtNDPK into isolated mitochondria, prior and after proteinase K treatment, respectively. Lanes 4, 5, in vitro import in the presence of 10  $\mu\text{M}$  valinomycin, prior and after proteinase K treatment, respectively. Lane 6, in organello phosphorylated pea mtNDPK [1]. 'p' denotes the precursor form and 'm' indicates the mature form of protein.

protein. However, no such band could be detected during in vitro import experiments (figure 2). In vitro processing experiments using crude mitochondrial extracts [32], that successfully process the alternative oxidase precursor from soybean [33], did not process the NDPK precursor protein (data not shown).

#### 3.3. Protein targeting

In order to experimentally confirm the mitochondrial localisation and to study the import pathway of the pea mtNDPK, in vitro import assays were carried out. In vitro translation of the NDPK BlueScript subclone produced a single 27 kDa translation product (figure 2, lane 1). The size of the precursor protein corresponds well to the theoretical value of 26 kDa calculated from the deduced amino acid sequence. Upon incubation of the precursor protein with isolated pea leaf mitochondria, the presence of a 16.5 kDa band can be observed (figure 2, lane 2). The 16.5 kDa band was proteinase K protected and hence inside the mitochondria (figure 2, lane 3). Moreover, the in vitro imported 16.5 kDa protein co-migrates with in organello phosphorylated NDPK (figure 2, lane 6), allowing us to identify this protein as the mature NDPK. The in vitro import was sensitive to the addition of the ionophore valinomycin (figure 2, lanes 4 and 5) indicating the requirement for a membrane potential for import. This suggests that the import pathway of this intermembrane space located NDPK involves the inner membrane protein import machinery [34].

#### 3.4. Phylogenetic analysis

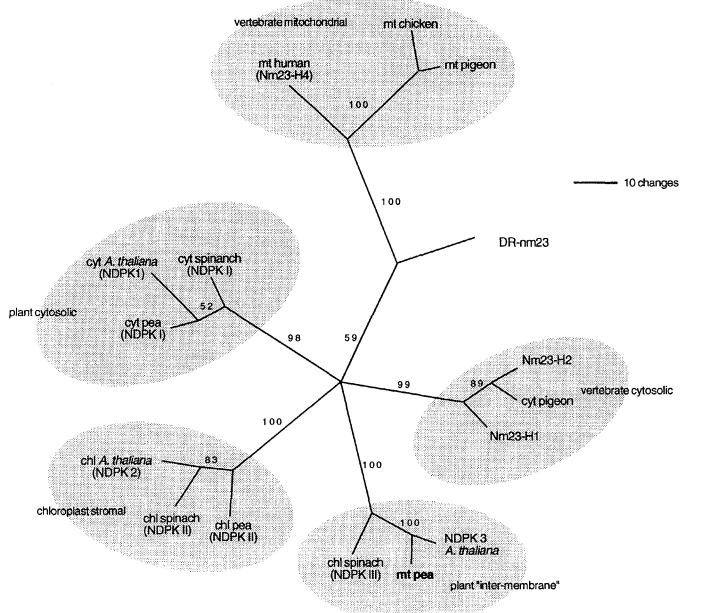
In order to investigate whether different evolutionary groups of NDPKs could be distinguished, and to place our

A

	-18	-4	-3	12	13	27	28	42	43	57	58	72
pea mtNDPK	-----	-----	▼	AELERTPTIAIKP	DGVORGLISEIISRF	ERKGFPLVGIKLVLP	TKQFAQQHYHDLKER	FFPFGLCDPLFSSGQV				72
<i>A. thaliana</i> NDPK3	-----	-----	-----	AEEMRTPTIAIKP	DGVORGLISEIISRF	ERKGFPLVGIKLVLP	SKDFPAQRHYHDLKER	FFPFGLCDPLFSSGQV				72
spinach NDPK III	-----	-----	-----	AEERTPTIAIKP	DGVORGLISEIISRF	ERKGFPLVGIKLVLP	SRDFPAQRHYHDLKER	FFPFGLCDPLFSSGQV				72
spinach NDPK II	-----	-----	-----	MEQVEETVIMIKP	DGVORGLVGEIISRF	EKKGFPLIGLKMYP	PKELAEHYHDLKAK	SFYQKLIQVITSGQV				73
<i>A. thaliana</i> NDPK 2	-----	-----	-----	MEQVEETVIMVAKP	DGIQRGLVGEIISRF	EKKGFPLIGLKMFC	PKELAEHYHDLKAK	SFFTLLEYITSGQV				73
pea NDPK II	-----	-----	-----	EQVDQAYIMVAKP	DGVORGLVGEIISRF	EKKGFPLVGIKLVLP	SKELAEHYHDLKAK	SFFPKLLEYITSGQV				72
pea NDPK I	-----	-----	-----	MAEQTFIMIKP	DGVORGLVGEIISRF	EKKGFPLVGIKLVLP	SRDFPAQRHYHDLKER	FFPFGLCDPLFSSGQV				71
spinach NDPK I	-----	-----	-----	MEQTFIMIKP	DGVORGLVGEIISRF	EKKGFPLVGIKLVLP	SRDFPAQRHYHDLKER	FFPFGLCDPLFSSGQV				70
<i>A. thaliana</i> NDPK 1	-----	-----	-----	MEQTFIMIKP	DGVORGLVGEIISRF	EKKGFPLVGIKLVLP	SRDFPAQRHYHDLKER	FFPFGLCDPLFSSGQV				70
pigeon NDPK	-----	-----	-----	MAANCERTPTIAIKP	DGVORGLVGEIISRF	EKKGFPLVGMKPVHA	SEELLKQHYHDLKDR	FFPFGLCDPLFSSGQV				74
human Nm23-H2	-----	-----	-----	MANLERTPTIAIKP	DGVORGLVGEIISRF	EKKGFPLVGMKPLFA	SEELLKQHYHDLKDR	FFPFGLCDPLFSSGQV				73
human Nm23-H1	-----	-----	-----	MANLERTPTIAIKP	DGVORGLVGEIISRF	EKKGFPLVGMKPLFA	SEELLKQHYHDLKDR	FFPFGLCDPLFSSGQV				73
human DR-nm23	-----	-----	-----	MAHCERTPTIAIKP	DGVORGLVGEIISRF	EKKGFPLVGMKPLFA	SEELLKQHYHDLKDR	FFPFGLCDPLFSSGQV				73
pigeon mtNDPK	-----	-----	-----	MICLVLTIIPANLFP	ACTGSAHERPTIAIKP	ERKGFPLVAMKLVGS	SEELLREHYALER	FFYGRLLVYMSGQV				90
chicken mtNDPK	-----	-----	-----	PELQEKTLVAVKVP	DAVORRLVGNVIRP	ERRGFPLVAMKLVGS	SEELLREHYALER	FFYGRLLVYMSGQV				73
human Nm23-H4	-----	-----	-----	GSAPPELRQTLVAVKVP	DAVORRLVGNVIRP	ERRGFPLVAMKLVGS	DRGLLDRHYQLQK	SFFYPALLAYMTSGEL				77
	-----	-----	-----	PSWTRERTLAVAKP	DGVORRLVGNVIRP	ERRGFPLVAMKLVGS	PESVLAERHYQDLRRK	SFFYPALLIRYMSGQV				74
				▲	KP D VQR L	RF E GF L K	* HYQ	F L	SGFV			74

	73	87	88	102	103	117	118	132	133	147	148
pea mtNDPK	IAMVWERKGVITVGR	KLIGATDPQKSAEPT		IRGDLAVVGNRIIH		GSDFPETARKEIKLV		FKPEELVSPVTSNEK		WIYGEN	▼
<i>A. thaliana</i> NDPK3	IAMVWEGDGVITVGR	KLIGATDPQKSEPT		IRGDLAVVGNRIIH		GSDFPETARKEIKLV		FKPEELVSPVTSNEK		WIYGEN	153
spinach NDPK III	IAMVWEGDGVITVGR	KLIGATDPQKSEPT		IRGDLAVVGNRIIH		GSDFPETARKEIKLV		FKPEELVSPVTSNEK		WIYGEN	153
spinach NDPK II	VCMAMWEGVAVASR	KLIGATDPLQAEPT		IRGDLAVVGNRIIH		GSDFPDKAKREIGLV		FKEGELCQWTPAQAP		WLIRE--	152
<i>A. thaliana</i> NDPK 2	VCMAMWEGVAVASR	KLIGATDPLQAEPT		IRGDLAVVGNRIIH		GSDFPDKAKREIGLV		FKEGELCQWTPAQAP		WLIRE--	152
pea NDPK II	VSMAMWEGVAVASR	KLIGATDPLQAEPT		IRGDLAVVGNRIIH		GSDFPDKAKREIGLV		FKEGELCQWTPAQAP		WLIRE--	151
pea NDPK I	VAMVWEGKVVITVGR	KLIGATNPAASEPT		IRGDFALDIGNRVIIH		GSDFVSAARKEIKLV		FKPEELVSPVTSNEK		WIY--	149
spinach NDPK I	VAMVWEGKVVITVGR	KLIGATNPAASEPT		IRGDFALDIGNRVIIH		GSDFVSAARKEIKLV		FKPEELVSPVTSNEK		WIY--	148
<i>A. thaliana</i> NDPK 1	VAMVWEGKVVITVGR	KLIGATNPAASEPT		IRGDFALDIGNRVIIH		GSDFVSAARKEIKLV		FKPEELVSPVTSNEK		WIY--	148
pigeon NDPK	VAMVWEGKVVITVGR	VMLGETNPADSKEPT		IRGDFCIQVGRNIH		GSDFVSAARKEIKLV		FKPEELVSPVTSNEK		WIY--	153
human Nm23-H2	VAMVWEGKVVITVGR	VMLGETNPADSKEPT		IRGDFCIQVGRNIH		GSDFVSAARKEIKLV		FKPEELVSPVTSNEK		WIY--	152
human Nm23-H1	VAMVWEGKVVITVGR	VMLGETNPADSKEPT		IRGDFCIQVGRNIH		GSDFVSAARKEIKLV		FKPEELVSPVTSNEK		WIY--	152
human DR-nm23	VAMVWEGKVVITVGR	ALIGATNPADAPPT		IRGDFCIQVGRNIH		GSDFVSAARKEIKLV		FKPEELVSPVTSNEK		WIY--	152
pigeon mtNDPK	VAMVWEGKVVITVGR	AMVGDTSQVAAAGT		IRGDFSMHVSRIIVH		ASDVSVAARKEIKLV		FKPEELVSPVTSNEK		WIY--	153
chicken mtNDPK	VAMVWEGKVVITVGR	AMVGDTSQVAAAGT		IRGDFSMHVSRIIVH		ASDVSVAARKEIKLV		FKPEELVSPVTSNEK		WIY--	157
human Nm23-H4	VAMVWEGKVVITVGR	AMVGDTSQVAAAGT		IRGDFSMHVSRIIVH		ASDVSVAARKEIKLV		FKPEELVSPVTSNEK		WIY--	155
	M W G	R	G T * A	G T	IRGD	N	H	SD	E I	W F	

B



protein among them, an alignment with other NDPK isoforms from the database was performed (figure 3A). Sequences from *A. thaliana*, pea, spinach, human, pigeon and chicken were used. These are all the higher eukaryotic species where sequences of NDPK isoforms representing different subcellular compartments are available.

The active site of the NDPKs is highly conserved and the phospho-histidine intermediate, identified by Gilles et al. [35] corresponds to His 117 in our sequence (figure 3A). Serine phosphorylation has been reported to be correlated with regulatory functions independent of the catalytic activity of the enzyme [3, 36]. Among the analysed sequences, only the serines at positions 69 and 119 are completely conserved. The residue corresponding to Ser44 in human Nm23-H1, which is the major serine phosphorylation site [3] is not conserved and in our sequence is replaced by a threonine. Therefore, the serine phosphorylation detected by Struglics and Håkansson [1] in the purified enzyme must reflect alternative phosphorylation sites, such as Ser119.

A clear difference between the vertebrate mitochondrial sequences and all the other analysed sequences is the absence of Pro95 in the former group. Pro95 has been shown to contribute to the stability of the hexameric oligomerisation of Nm23-H2 [37]. The mutation Pro-Ser at this position, so-called *killer of prune* in *Drosophila* [6] is lethal in the genetic context of the *prune* eye colour mutation, but has no effect on the catalytic activity of the enzyme. Hexamerisation has also been associated with the YE motif at the C-terminus of the cytosolic isoforms. The YE motif is found in all cytosolic isoforms but in none of the organellar isoforms, where other sequences are present. However, the chloroplastic NDPK II C-terminal RE motif is evidently compatible with hexameric structure, since this has been observed in spinach [38]. Prokaryotic NDPKs have been reported to form tetramers [39] and although there is no direct evidence for tetramerisation of isoforms with other C-terminal motifs, such as the GDN found in the pea mtNDPK sequence (figure 3A), it remains a possibility.

The amino acid sequences were trimmed to flush conserved ends prior to phylogenetic analysis as indicated in figure 3A. This alignment was used in the construction of a phylogenetic tree by the maximum parsimony method [25]. The tree is shown in figure 3B as an unrooted phylogram representation of the bootstrap consensus tree

of two topologically identical trees (cut at 50% confidence). Five distinct groups can be observed. They are associated with their subcellular localisation and origin (vertebrate mitochondrial, vertebrate cytosolic, plant 'inter-membrane', chloroplast stromal and plant cytosolic isoforms). The pea mitochondrial NDPK groups together with a putative mitochondrial *Arabidopsis* sequence and the NDPK III isoform found in spinach chloroplasts. The pea mtNDPK seems not to be more closely related to the vertebrate mitochondrial group than to any other group. Other methods such as the UGMPA and neighbour-joining as implemented in the PAUP program package were also employed and resulted in identical phylogenetic topologies but with differing support values (data not shown).

Two interesting exceptions from the grouping in the phylogenetic tree are the cytosolic DR-nm23 [40] from human and the chloroplastic NDPK III from spinach. The spinach NDPK III is placed together with two plant mitochondrial NDPKs while the DR-nm23 can not be confidently placed in either the vertebrate cytosolic or the mitochondrial group.

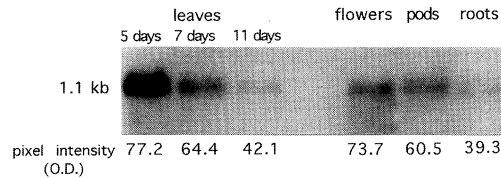
The deduced amino acid sequence of the mature pea mtNDPK showed a 90% (138/153) identity with the deduced sequence of a putative mitochondrial NDPK gene from *A. thaliana*, *ndpk3*. A 58.8% identity was observed when only the targeting sequences deduced from these two clones were compared. The same overall secondary structure is predicted for the *A. thaliana* and the pea mtNDPK targeting sequences [28].

### 3.5. Expression analysis

The expression of genes coding for organelle proteins is spatially and temporary regulated [41-43]. We followed the expression pattern of the pea mtNDPK mRNA in different parts of the plant as well as at various developmental stages of pea leaves. Samples were collected from budding leaves (5 days old), through expanding leaves (7-9 days old), fully expanded leaves (11-21 days old), to senescent leaves (more than 21 days old). The Northern blot analysis shows in all cases a single transcript with an estimated size of 1.1 kb (figure 4).

From the studied tissues we can conclude that the expression of this gene is higher in reproductive organs (flowers and pods) than in vegetative tissues (fully ex-

**Figure 3. A.** Amino acid sequence alignment of the pea mtNDPK and some representative NDPKs. Completely conserved amino acids are indicated below the alignment, Ser67 and Ser119, which are mentioned in the text are presented in bold. Asterisks mark other residues discussed in the text. The place where the sequences were trimmed to flush conserved ends prior to phylogenetic analysis are indicated with arrowheads. The aligned sequences with accession number are listed below: Nm23-H1 (P15531), Nm23-H2 (P22392), DR-nm23 (Q13232), Nm23-H4 (O00746) from human; NDPK I (Q02254), NDPK II (Q01402), NDPK III (S60363) from spinach; mtNDPK (AF191098), NDPK I (P47922), NDPK II (P47923) from pea; NDPK 1 (P39207), NDPK 2 (O64903), NDPK 3 (AF044265) from *A. thaliana*; mNDPK (P87355), NDPK (Q90380) from pigeon and mNDPK (AF043542) from chicken. **B.** Unrooted phylogram representation of the consensus phylogenetic tree constructed by the maximum parsimony method with indicated bootstrap values on the branches. The sequences were trimmed to flush conserved ends prior to phylogenetic analysis.



**Figure 4.** Analysis of the expression of the pea mtNDPK. For comparison among tissues the fully expanded leaves (11 days old) were taken as representatives of leaf tissue. Approximately 10 µg of total RNA were loaded per lane. The intensity of the hybridisation signal is given in numbers under each lane (numbers were adjusted to the differences in loading, using the 26S rRNA as standard). Data were analysed using ImageQuant 1.2 software.

panded leaves and roots). This tissue specific expression follows the pattern of other nuclear-encoded mitochondrial proteins [44, 45]. Furthermore, we observed a higher expression in young leaves as compared to old ones. The observed decrease of transcript levels with age continued through to day 21 (data not shown). This could be interpreted as a reflection of declining mitochondrial activity in mature leaves, where respiration has been shown to decrease once the tissue becomes more photosynthetically active [46, 47].

Stress conditions have been shown to affect expression of some NDPK isoforms [9, 48, 49]. RNA was isolated from leaves of pea plants exposed to high salt conditions and to low or high temperature. However, none of the treatments showed any effect on the expression pattern of the pea mitochondrial NDPK (results not shown).

#### 4. Discussion

As a first step in characterising a possible signal transduction pathway in pea mitochondria, a cDNA clone encoding a member of the highly conserved Nm23/NDPK family was isolated. Targeting of the *in vitro* translation product into isolated mitochondria confirmed its organellar localisation. According to the literature, four mechanistically different pathways have been established for nuclearly encoded mitochondrial proteins to reach the intermembrane space (IMS): i) receptor-independent, reversible passage over the outer membrane followed by co-factor ligation, e.g., cytochrome *c* [50]; ii) receptor-dependent with no cleavable targeting sequence, e.g., the heme lyases [51]; iii) conservative sorting: complete translocation into the matrix, removal of targeting sequence, followed by export over the inner membrane to the IMS, e.g., the Rieske protein [34]; and iv) stop-transfer model: engagement with the inner membrane protein

import machinery, removal of matrix targeting sequence, stop-signal in a hydrophobic helix followed by lateral movement in the membrane for further maturation by a signal peptidase, e.g., cytochrome *b*<sub>2</sub> [52]. Our results suggest that the import pathway of the pea mtNDPK follows either route iii or iv. Firstly, the protein has a targeting sequence that is removed upon import into the mitochondria. Secondly, the import is dependent on a membrane potential, indicating the involvement of the inner membrane protein import machinery.

The lack of processing of the mtNDPK precursor during the *in vitro* assays suggests an unusual processing of this protein. Moreover, the absence of any MPP recognition site may indicate that the processing observed during *in vitro* import is not due to MPP but possibly to a functional homologue to inner membrane peptidase (IMP) as described by Nunnari et al. [53]. The IMP belongs to the signal peptidase family as does the analogous thylakoid processing peptidase (TPP). The specificity of the TPP is such that it cleaves after short chain residues at the -3 and -1 positions where alanine at the -1 position is essential [54]. In our sequence a valine is located at position -3 and an alanine at position -1 of the *N*-terminus of the mature protein. By analogy to the TPP processing activity we can describe the pea mtNDPK targeting sequence as a good candidate substrate for a putative plant mitochondrial signal peptidase.

A very high sequence similarity was found between the pea mtNDPK cDNA and *ndpk3* from *A. thaliana*. A high similarity was also found between the deduced targeting sequences of the two proteins. In addition, the absence of any MPP cleavage site as well as the presence of a possible TPP-like recognition sites are features of both the pea mtNDPK and the *A. thaliana* targeting sequences. Given the high similarity between the two sequences, we would suggest that these two proteins are not only sharing the same function, but also the same subcellular localisation and import pathways. This was supported by the phylogenetic analysis where the pea mtNDPK and the *A. thaliana* NDPK3 group together.

The spinach NDPK III sequence was determined from direct sequence of the isolated protein [12] and therefore no information concerning its targeting sequence is available. This soluble isoform has been found in low abundance, as compared to the stromal NDPK II, in purified chloroplasts [38]. The high degree of similarity with the plant mitochondrial isoforms makes it tempting to propose an inter-envelope localisation of the spinach NDPK III, being placed in an analogous position to the intermembrane space pea mitochondrial isoform. Inter-membrane NDPKs have been suggested to play an important role in the nucleoside balance between organelles and cytosol [13]. Future identification of proteins interacting with the pea mitochondrial NDPK may help to unravel a potential role of this protein in intracellular communication.



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*IV*



## Heat stress response in pea involves interaction of mitochondrial nucleoside diphosphate kinase (mtNDPK) with a novel 86 kDa protein

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### Abstract

In this work we have further characterised the first mitochondrial nucleoside diphosphate kinase (mtNDPK) isolated from plants. The mitochondrial isoform was found to be especially abundant in reproductive and young tissues. Expression of the pea mtNDPK was not affected upon different stress conditions. However, the pea mtNDPK was found to interact with a novel 86 kDa protein, which is *de novo* synthesised in pea leaves upon exposure to heat. Thus, we have evidence for the involvement of mtNDPK in mitochondrial heat response in pea *in vivo*. Studies on oligomerisation of the pea mtNDPK revealed that this mitochondrial isoform, like other organellar isoforms, can form hexamers. In addition to the hexamers, the protein was also found as tetramers and dimers displaying flexibility in oligomerisation. This flexibility, also found for other NDPK isoforms, has been correlated with the ability of this enzyme to interact with other proteins. We believe that the mtNDPK is involved in heat stress response in pea, possibly as a modulator of the 86 kDa protein.

## Introduction

Nucleoside diphosphate kinases (NDPKs) are ubiquitous enzymes that transfer phosphate groups from triphosphate nucleosides (NTPs) to nucleoside diphosphates (NDPs) (Parks and Agarwal, 1973). Besides this house-keeping function, recent reports have revealed the involvement of animal NDPKs in vital processes such as control of cell proliferation (Cipollini *et al.*, 1997), regulation of transcription (Ji *et al.*, 1995; Postel *et al.*, 1993) and protein phosphotransferase activity (Engel *et al.*, 1998; Wagner and Vu, 2000). In plants, phytochrome B response (Choi *et al.*, 1999), UV-B light signalling (Zimmermann *et al.*, 1999) and hormone responses (Nato *et al.*, 1997; Novikova *et al.*, 1999) are among the processes in which NDPK isoforms have been shown to be involved.

The oligomeric structure of NDPKs has been shown to be important for the function of this enzyme. With the exception of some prokaryotic NDPKs, that are tetrameric, most of the known NDPK isoforms are active as hexamers, including the chloroplastic NDPK II from spinach (Yang and Lamppa, 1996) and the human mitochondrial Nm23-H4 (Milon *et al.*, 2000). However, the organellar isoforms lack the typical C-terminus motif that is believed to stabilise the hexameric conformation of cytosolic isoforms (Webb *et al.*, 1995). Correlation between the stability of the NDPK's oligomeric structure and its function has been found *in vitro* in *Dictyostelium* (Mesnildrey *et al.*, 1997). While the wild-type hexameric NDPK lacks DNA-binding activity, point mutations, that cause destabilisation of the hexamer structure lead to dimer formation and enables this mutant protein to bind DNA.

Interactions of NDPKs with different types of proteins have been reported in several cases. For example, in human erythrocytes, dimers of nm23-H1 and of glyceraldehyde-3-phosphate dehydrogenase form a hetero-tetrameric complex, that has serine/threonine phosphotransferase activity (Engel *et al.*, 1998). In *Arabidopsis thaliana*, interaction between phytochrome B and NDPK 2 has been shown to occur (Choi *et al.*, 1999). Also commonly found but less studied is the interaction of NDPK with heat shock proteins. In *Poeciliopsis lucida* a 16 kDa NDPK modulates the activity of Hsc70 (Leung and Hightower, 1997). Moreover, in *E. coli* the co-purification NDPK and Dna K, a Hsp70 protein, suggests a similar type of interaction (Barthel and Walker, 1999).

NDPK isoforms have been found in the matrix as well as the inter-membrane space of mitochondria (Lambeth *et al.*, 1997; Milon *et al.*, 1997; Struglics and Håkansson, 1999; Troll *et al.*, 1993). In animals, matrix NDPK isoforms have been suggested to catalyse transfer of the phosphoryl group from GTP, produced by the TCA cycle, to ATP (Herbert *et al.*, 1955). However, in plants no GTP is directly generated by the TCA cycle (Palmer and Wedding, 1966). Early reports suggested that the function of an inter-membrane isoform would be generation of triphosphate nucleosides needed in the cytosol, using the ATP produced by the mitochondrion as substrate (Pedersen, 1973). However, the functions of the different isoforms remain under debate. Struglics and Håkansson (1999) purified the first plant

mitochondrial isoform and suggested an inter-membrane space localisation. This 17 kDa isoform, purified from pea mitochondria, shows auto-phosphorylation on serine residues, a characteristic of the human NDPK isoforms involved in signal transduction (McDonald *et al.*, 1993; Postel *et al.*, 1993). Sequence and phylogenetic analysis of the cDNA encoding for this mitochondrial isoform (Escobar Galvis *et al.*, 1999) revealed high similarity with NDPK3 from *Arabidopsis thaliana* and the chloroplastic NDK III from spinach. The similarity among these isoforms might reflect an analogous localisation within the organelles: the mitochondrial inter-membrane space and the inter-envelope in chloroplasts. We believe that, due to its sub-cellular localisation and biochemical features (Struglics and Håkansson, 1999), the pea mitochondrial NDPK (mtNDPK) possibly acts as a signalling element between mitochondria and cytosol.

Expression of NDPKs varies between different tissues and developmental stages. In wheat grains, NDPK protein levels were found to be more abundant in the embryo than in pericarp-testa (Hurkman *et al.*, 1998). The pea mtNDPK showed higher transcript levels in young leaves and reproductive tissues as compared to mature leaves and roots (Escobar Galvis *et al.*, 1999). In addition, stress conditions such as wounding in tomato has been shown to affect transcription of an NDPK isoform (Harris *et al.*, 1994). Moreover, phosphorylation of a NDPK isoform was affected by heat stress in sugarcane (Moisyadi *et al.*, 1994). Even though, heat-stress response in plants have been shown to involve proteins such as NDPK (Moisyadi *et al.*, 1994) and BiP (binding protein) (Hurkman *et al.*, 1998), studies on heat-stress response in plant mitochondria have been mainly focused on characterisation of heat shock proteins (HSPs) (Neumann *et al.*, 1993; Lund *et al.*, 1998). Only one mitochondrial small heat shock protein, which provides thermotolerance to respiratory complex I (Downs and Heckathorn, 1998), have been functionally studied. Investigations of the possible involvement of other proteins, such as NDPK, in mitochondrial response to heat stress are therefore relevant.

The purpose of this work was to functionally characterise the pea mtNDPK, investigating tissue specificity in expression as well as a possible role in response to different kinds of stress. In this paper, by using immunocytochemistry, we show a higher expression of the pea mtNDPK protein in young leaves and the reproductive parts of the flower bud as compared to mature leaves and vegetative parts of the flower. We also report a novel interaction of a 86 kDa protein with the pea mtNDPK under heat stress, providing evidence for a role of this mitochondrial NDPK isoform in stress response in plants. In addition, studies on the oligomerisation of the pea mtNDPK revealed that this protein can form hexamers as well as tetramers.

## Results

### *Tissue-specific expression of the pea mtNDPK*

In order to avoid cross-reaction of the mtNDPK antibody with other NDPK isoforms, we selected a region at the C-terminus of the protein for antibody production. This region has no similarities with other plant NDPKs (Escobar Galvis *et al.*, 1999). When isolated mitochondria were probed with the antibody, only one band (16.5 kDa) in the region corresponding to NDPK isoforms (around 17 kDa) was detected (Figure 1, lanes 1-4). The size of the detected protein corresponds to the size of the purified mitochondrial NDPK (lane 5, supplied by Struglics and Håkansson). No cross-reaction of the anti-mtNDPK with any of the chloroplast isoforms was observed (lane 6), demonstrating the specificity of the antibody.

We were able to detect the presence of the mtNDPK in isolated mitochondria from flowers, leaves (7 and 9 days old) and roots using western analysis. No significant differences were observed when mitochondria isolated from the different tissues were compared (Figure 1, lanes 1-4). Based on these observations we concluded that the steady state level of pea mtNDPK does not seem to vary among mitochondria of different origin.

On the other hand, when whole tissues probed with anti-mtNDPK were studied by light microscopy clear differences in signal were observed within each tissue as well as between leaves of different developmental stages. In flower buds (Figure 2, panel A), the signal specifically corresponding to anti-mtNDPK was higher in central and lateral parts of the bud than in the outer layers of tissue. When compared to the sample incubated only with the pre-immune serum (panel B), it is clear that the anti-mtNDPK specifically reacts only with ovary, stamen and petal tissues (panel A). When the anthers are observed in more detail (panel C), and compared to the pre-immune serum sample (panel D), the labelling is found mainly in the cytoplasm (which contains the mitochondria) of the inner cells. No label is present in the vacuole. When sections of young leaves (non-expanded leaves) were labelled we were able to see that the mtNDPK is preferentially localised in the lower mesophyll (Figure 2, panel E). A tissue that later contains large air space and lower chloroplast number. In the sample probed with the pre-immune serum, no such specific localisation of label is seen (panel F). Conversely, in expanded leaves, a photosynthetically active tissue, mtNDPK seems to be found in lower amounts and with no preference for any cell type (results not shown).

### *A 86 kDa protein is newly synthesised upon heat-stress and co-precipitates with the pea mtNDPK*

Previous studies have shown involvement of NDPK isoforms in stress responses (Harris *et al.*, 1994; Zimmermann *et al.*, 1999). We therefore investigated mtNDPK expression after exposure to stress conditions such as high salt (400 mM NaCl), oxidative (2% H<sub>2</sub>O<sub>2</sub>), cold (4°C) and heat (42°C). Western analyses confirmed the presence of mtNDPK in the different



samples (Figure 3), showing decreased levels at 42°C (lane 4) as compared to the control (lane 1). Decreased levels of mtNDPK were also found in the presence of high salt (lane 2) or H<sub>2</sub>O<sub>2</sub> (lane 3), probably a result of increased proteolytic activity upon exposure to all these types of stress.

In order to detect whether the mtNDPK was *de novo* synthesised under stress conditions, pea seedlings were supplied with [<sup>35</sup>S]methionine during cold and heat stress. Immunoprecipitation assays of solubilised proteins isolated from crude mitochondria, with the mtNDPK antibody, were carried out to detect differential expression of this protein upon stress. No band corresponding to the 17 kDa pea mtNDPK was <sup>35</sup>S-labelled (Figure 4A, lanes 1-3) showing that mtNDPK expression does not seem to be up-regulated during the stress conditions tested here. However, a single labelled 86 kDa band (lane 2) was observed in seedlings that had been exposed to heat stress (4 hr at 42°C). Incorporation of [<sup>35</sup>S]methionine into the 86 kDa protein was observed after 2 hours of heat treatment increasing in a time-dependent manner for 8 hours (Figure 4B, lanes 1-3). The 86 kDa protein co-precipitates with the mtNDPK suggesting a specific interaction between the two proteins. In addition, the results indicate involvement of the pea mtNDPK in mitochondrial heat-stress response.

Parallel assays were carried out in the absence of the isotope in order to check the efficiency of the immunoprecipitation assay. Gel blots of the immunoprecipitation assays probed with the mtNDPK anti-serum confirmed the presence of NDPK in all samples (Figure 4C, lanes 1-5). These results show that the absence of a <sup>35</sup>S-labelled band corresponding to the mtNDPK is not due to failure of the immunoprecipitation assay. Rather we can conclude that mtNDPK is not newly synthesised upon cold or heat stress conditions, in agreement with the results from the western analysis (Figure 1).

#### *The 86 kDa protein is a novel protein*

The [<sup>35</sup>S]methionine labelled 86 kDa protein was also detectable by coomassie staining of the SDS-PAGE gels, allowing sequencing of this heat up-regulated protein. Unfortunately, *N*-terminal sequencing of the 86 kDa protein was inconclusive, due to *N*-terminal blocking of this protein. Using mass spectrometry sequences of trypsin digested peptides were obtained, however analysis of the sequences revealed no similarity with other proteins found in the databases, making identification of the 86 kDa heat up-regulated protein impossible.

#### *The mtNDPK can be found in various oligomeric states*

Taking into account the reports of correlation between function and structure of the NDPKs in animals (Mesnildrey *et al.*, 1998; Mesnildrey *et al.*, 1997) we wanted to determine the oligomeric structure of this mitochondrial isoform. After gel filtration of mitochondrial soluble

proteins and immunodetection, the pea mtNDPK was detected in several fractions with peaks corresponding to approximately 100, 80, 60, 45, 30 and 15 kDa (figure 5). The peaks approximately correspond to calculated sizes of different oligomeric states of mtNDPK: monomer (15 kDa), dimer (30 kDa), trimer (45 kDa), tetramer (60 kDa), pentamer (80 kDa) and hexamer (100 kDa).

## Discussion

Western analysis revealed no differences in the amounts of mtNDPK compared to total mitochondrial protein in the various tissues studied in this work. On the other hand, immunolocalisation studies revealed high levels of mtNDPK in flower and young leaves, tissues which require high mitochondrial activity to cover their energy requirements (Moneger *et al.*, 1994; Smart *et al.*, 1994; Thompson *et al.*, 1998). The higher mitochondrial activity in these tissues could be explained by a higher number of mitochondria (Huang *et al.*, 1994). The results obtained by immunolocalisation of the pea mtNDPK are in agreement with previous studies using northern analysis (Escobar Galvis *et al.*, 1999), where higher levels of mtNDPK mRNA were found in young and reproductive tissues. Taking our new observations into account we can conclude that the differences in pea mtNDPK expression previously reported are likely due to differences in mitochondrial number among the studied tissues.

In young non-expanded pea leaves, the pea mtNDPK was preferentially found in the lower mesophyll, a tissue that is known to contain less chloroplasts than its upper counterpart. Since the lower mesophyll is a less photosynthetically active tissue, respiration must be an important source of metabolites and ATP. Furthermore, when mature leaves were studied, the amounts of mtNDPK were found to be very low, probably an indication of lower mitochondrial activity or mitochondrial number. These results are in agreement with previous work showing that the levels of respiratory capacity and efficiency of oxidative phosphorylation decreases with ageing in pea leaves (Azcón-Bieto *et al.*, 1983).

Flower buds showed high amounts of mtNDPK localised to the central and lateral parts of the bud, whereas in the peripheral parts the signal was comparable to the sample incubated with the pre-immune serum. Development of pea flowers have been shown to be a complex process that involves the presence of common primordia to petals and stamens (Ferrandiz *et al.*, 1999). This peculiarity would explain that tissues like petals and stamens showed similar levels of mtNDPK at this early stage of development (Figure 2, panel A). In sunflower (Smart *et al.*, 1994), mitochondrial gene expression has been found to be correlated with flower development. Smart and co-workers showed that the mitochondrial  $\alpha$  subunit of the F<sub>1</sub>-ATP synthase was most abundant in young meiotic cells in anthers, a tissue responsible for the development of haploid microspore cells. This process has a high energy demand that can only be covered by high mitochondrial activity and/or increased mitochondrial number.

The *in vivo* labelling assays showed that upon heat stress the pea mtNDPK interacts with a newly synthesised 86 kDa protein. The results indicate the involvement of the mtNDPK in

heat-stress response. Previous work has shown that modulation of protein activity by interaction with NDPK isoforms occurs. In the fish *Poeciliopsis lucida* NDPK controls oligomerisation of Hsc70, thereby affecting its activity (Leung and Hightower, 1997). It would be plausible to suggest that the pea mtNDPK could act as a modulator of the heat up-regulated 86 kDa protein. The mechanism of such a regulation remains to be investigated. Besides control of oligomerisation, the phospho-transferase activity reported for some NDPK isoforms (Engel *et al.*, 1998; Engel *et al.*, 1995; Wagner *et al.*, 1997; Wagner and Vu, 2000) could regulate activity other proteins.

Determination of the oligomeric state of the mtNDPK revealed the presence of this mitochondrial isoform in several fractions corresponding to a wide range of molecular sizes (100, 80, 60, 45, 30 and 15 kDa). It is possible that these fractions represent a step-wise oligomerisation from monomer to hexamer. On the other hand, it has been proposed for the *Dictyostelium* NDPK (Mesnildrey *et al.*, 1998) that hexamerisation is the result of trimerisation of dimers that would need the formation of a tetramer intermediate. Formation of the intermediate tetramer requires the presence of nucleoside substrate (e. g. ATP) after which hexamerisation is possible. It was further proposed that interaction of NDPK with other proteins could occur at the tetramer intermediate state, where the structure would allow interaction with a larger substrate. One cannot exclude that such interactions of the pea mtNDPK with other soluble proteins could be detected through gel filtration. For example, in *E. coli* Dna K and NDPK have been shown to co-purify (Barthel and Walker, 1999). An analogous interaction between the mtNDPK and the mitochondrial hsp70 could explain the peak observed around 80 kDa.

In conclusion, we have shown that the pea mtNDPK is probably involved in heat-stress response, due to its interaction with the 86 kDa protein. We propose that the pea mtNDPK might act as a modulator of the 86 kDa protein being part of the mitochondrial heat response in higher plants. It is possible, taking into account the different oligomeric states detected for this protein, that the pea mtNDPK can interact with various additional substrates.

## Materials and methods

*Plant material.* Garden peas (*Pisum sativum* L. cv Oregon sugarpod) were grown, on vermiculite in a growth chamber at 20°C with a 12 hours day (25  $\mu\text{Em}^{-2}\text{s}^{-1}$ ).

*Mitochondria isolation.* Six g of pea leaves from 12-13 days old seedlings were incubated for 4 hours at 4°C or at 42°C. The seedlings were ground with a polytron (9500 rpm) in 40 ml ice-cold homogenisation buffer (0.4 M sucrose, 50 mM Tris, 1 mM EGTA and 10 mM  $\text{KH}_2\text{PO}_4$ , 5 mM 2-mercaptoethanol, 1% (w/v) BSA, 0.1% (w/v) PVP-44, pH 7.6). The resulting supernatant, after centrifugation at 3100g for 2 min, was spun at 15 300g for 15 min. The obtained pellet was used as crude mitochondria. Percoll (Pharmacia-Biotech, Uppsala, Sweden) purified mitochondria were isolated as in Boutry *et al.* (1984) with modifications

according to Håkansson *et al.* (1998). As a control mitochondria were isolated from seedlings kept at room temperature (22°C) for 4 hours.

*Chloroplasts isolation.* Chloroplasts were prepared from 12-13 days old pea leaves according to Walker (1971).

*Antibody.* A peptide corresponding to the last 15 amino acids of the C-terminus of the pea mitochondrial NDPK (accession number AF191098) was synthesised and conjugated to keyhole limpet hemocyanin using maleimide crosslinker. Innovagen (Lund, Sweden) produced a rabbit polyclonal antiserum raised against this peptide. Anti-mtNDPK was used in a 1:5000 dilution for western analysis and 1:50 for immunolocalisation experiments.

*Western blotting.* Proteins were separated by SDS-PAGE (Laemmli, 1970) using BioRad's mini-gel system and transferred onto nitrocellulose membranes (Hybond ECL, Amersham International, Buckinghamshire, UK). Transfer was performed in a Multiphore II NovoBlot unit (Pharmacia, Sweden) using the transfer buffer of Bjerrum and Shafer-Nielsen (1986) (48 mM Tris, 39 mM glycine, 20% (v/v) methanol) at 5 mA·cm<sup>-2</sup> for 30 min. Membranes were blocked in 3% (w/v) BSA (fraction V, Sigma) in TBS (100 mM Tris, 150 mM NaCl) at 4°C, overnight. Incubation with the primary antibody (diluted in 3% (w/v) BSA in TBST (TBS containing 0.1% (v/v) Tween-20) was carried out for at least 4 hours at room temperature. The secondary antibody, an alkaline phosphatase conjugated goat anti-rabbit (Bio-Rad Laboratories, Hercules, CA, USA) was used as a 1:10 000 dilution and the protein was detected using the Immun-Star Chemiluminiscent Protein Detection Systems (Bio-Rad Laboratories, Hercules, CA, USA) following manufacturers' instructions.

*Immunocytochemistry.* Semi-thin sections were prepared from purified pea mitochondria, pea flower buds, young (6 days old) and mature leaves (fully expanded, 11 days old) as in Marttila *et al.*, (1996). Briefly, the material was fixed in 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in PBS (10 mM Na-phosphate buffer pH 7.4, 150 mM NaCl) for 2 hours at room temperature, dehydrated, infiltrated with medium-grade London Resin White (London Resin Company Ltd., Reading, UK distributed by TAAB), and polymerised for 24 h at 58°C. Sections of 1 µm were cut on Superfrost Plus slides (Menzel Gläser, Germany) and blocked in 5% (v/v) goat normal serum and 1% (w/v) BSA in PBS for 30 min at room temperature. Primary antibody incubation with anti-mtNDPK (1:50 in 1% (w/v) BSA in PBS) was carried out at 4°C overnight. After 4 washes in PBS for 20 min, the sections were incubated with a goat-anti-rabbit IgG conjugated to FITC (Sigma) for 1 hour at 37°C. On control slides, either pre-immune serum was used instead of the primary antibody, or both primary and secondary antibody were replaced with dilution buffer. Samples were washed with PBS and water and mounted in 20% Mowiol 4-88 (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) in PBS pH 8.6 containing 0.1% phenylenediamine. Immunofluorescence was investigated under UV light (excitation filter 495 nm, barrier filter 520 nm) in a Leica microscope.

*In vivo* labelling under stress conditions. Six to eight (12-13 days old) seedlings were cut off and incubated for 4 hours at 4°C or at 42°C, in water containing 0.2 mCi of [<sup>35</sup>S]methionine (Amersham, SJ1015). The control sample was kept at room temperature (22°C) for 4 hours. After incubation the leaves were detached and ground in 5 ml ice cold homogenisation buffer together with 1 g of glass beads. All the following steps were carried out between 4-8°C. The homogenate was filtered through four layers of Miracloth (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) and spun for 3 min. at 2500 g in a microcentrifuge. The supernatant was then spun for 15 min at 16 200g. The resulting pellet was resuspended in 400 µl of solubilisation buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, 1 mM PMSF). A soluble fraction was obtained after centrifugation for 5 min. at 16 200g, the pellet was discarded. A time course at 42°C was carried out under the same conditions collecting samples every second hour during an 8 hour period.

*Immunoprecipitation.* 350 µl of the soluble fraction were incubated with the mitochondrial NDPK antibody (1:100 dilution) overnight at 4°C. Proteins were precipitated by addition of Protein G Sepharose 4 Fast Flow (Pharmacia-Biotech, Uppsala, Sweden). After 1 hour of incubation the beads were washed 3 times in solubilisation buffer (0,25% Nonidet P-40, 150 mM NaCl, 50 mM Tris, 1 mM PMSF) and once in washing buffer (50 mM Tris-HCl, pH 8.0) following manufacturers' instructions. The proteins were denaturated in reducing sample buffer (100 mM DTT, 1% SDS, 50 mM Tris, pH 7.5) by heating at 95°C for 3 min. Proteins were separated by SDS-PAGE (Laemmli, 1970) and gels were vacuum-dried and exposed to PhosphorImager plates (Molecular Dynamics, Sunnyvale, CA, USA). Data were analysed using ImageQuant 1.2 software (Molecular Dynamics).

*Protein sequencing.* For *N*-terminal sequencing, immunoprecipitation of crude mitochondrial soluble proteins prepared from pea leaves exposed to heat stress were separated by SDS-PAGE. After electroblotting onto PVDF membrane (Pall Europe Limited, Portsmouth, UK) proteins were detected via coomassie staining according to the manufacturers' instructions. For internal sequencing proteins prepared as above were separated by SDS-PAGE and briefly coomassie stained and destained. Sequence analysis (*N*-terminal sequencing, in-gel digestion, peptide extraction, mass mapping and tandem mass spectrometry) were performed by the Protein Analysis Center, Karolinska Institutet (Stockholm, Sweden). *N*-terminal sequencing was carried out as follows: the excised protein were applied to a Procise cLC sequencer (PE-Applied Biosystems, USA) for Edman degradation.

Internal sequencing was carried out as follows: the coomassie-stained protein band was cut from the gel and the piece was placed in an Eppendorf tube for in-gel digestion. Briefly, washing was carried out in 0.2 M ammonium bicarbonate containing 50% acetonitrile. The protein was reduced (DTT) and alkylated (iodoacetamide) followed by in-gel digestion with 0.5-3 µg trypsin (Promega, modified) in 0.2 M ammonium bicarbonate overnight at 37°C. The tryptic peptides were extracted using acetonitrile in 0.1% trifluoroacetic acid, first at 60% then at 40%. Aliquots of the peptide extract, were desalted (ZipTip C18, Millipore) and analysed by MALDI mass spectrometry (Voyager DE-PRO, Applied Biosystems). For protein

identification, the resulting mass map was analysed with computer algorithms (MS Fit, Pep Sea and Pro Found) in screens of internet accessible sequence databases. For tandem mass spectrometry analysis (Q-TOF, Micromass), aliquots were applied using a nano-electrospray ion source and argon as the collision gas. Amino acid sequence interpretation from peptide fragment data was aided by software supplied by Micromass.

*Gel filtration.* Purified mitochondria were osmotically ruptured in ice-cold 5 mM EDTA, in the presence of 200  $\mu$ M ATP. The resulting suspension was spun at 16 200g in a microcentrifuge for 15 min at 4°C, separating the membrane from the soluble fraction. Soluble mitochondrial proteins were separated in Sephacryl S-200 HR column (Pharmacia, Uppsala, Sweden) using PBS buffer (pH 7.3). The column was operated using the FPLC system (Pharmacia, Uppsala, Sweden) at a flow of 0.3 ml.min<sup>-1</sup>. The collected (1 ml) fractions were blotted onto nitrocellulose membranes (Hybond-ECL Amersham International, Buckinghamshire, UK), using a BIO-DOT SF slot-blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturers' instructions and probed with the anti-mtNDPK as in the western blotting analysis. Phosphorylase B (97.16 kDa), BSA (66 kDa), carbonic anhydrase (30 kDa) and cytochrome c (12.4 kDa), were used as standards.

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## Figure legends

### Figure 1

Western analysis of the pea mtNDPK in various subcellular fractions. Lane 1, flower mitochondria; lane 2, root mitochondria; lane 3, 7 days old leaves mitochondria; lane 4, 9 days old leaves mitochondria; lane 5, purified pea mtNDPK (according to Struglics and Håkansson, 1999) and lane 6, purified chloroplasts.

### Figure 2

Immunolocalisation of the pea mtNDPK in flower bud and young pea leaf. Positive fluorescent immunolabelling of the mtNDPK is seen as bright green spots. Panels A-D, flower bud; panels E-F non-expanded pea leaf. The pictures represent the following: A, longitudinal flower bud section, incubated with anti-mtNDPK; B, longitudinal flower bud section, incubated with pre-immune serum; C, transversal anthers section, incubated with anti-mtNDPK; D, transversal anthers section, incubated with pre-immune serum; E, transversal young pea leaf section, incubated with anti-mtNDPK; F, transversal young pea leaf section, incubated with pre-immune serum. A, anthers, O, ovary; ST, stamen; P, petals; SE, sepals; UM, upper mesophyll; LM, lower mesophyll; V, vein. Scale bars represent 100  $\mu$ m.

### Figure 3

Western analysis of the pea mtNDPK in crude mitochondria prepared from pea leaves exposed to various stress conditions for 4 hours. Lane 1, control; lane 2, high salt stress (400 mM NaCl); lane 3, oxidative stress (2% H<sub>2</sub>O<sub>2</sub>); lane 4, heat stress (42°C); lane 4, cold stress (4°C).

### Figure 4

Analysis of *de novo* synthesised protein in pea upon heat and cold stress.

Panel A. PhosphorImage of immunoprecipitation of [<sup>35</sup>S]methionine labelled crude mitochondrial proteins using the pea mtNDPK antibody. Lane 1, control; lane 2, heat stress (42°C) and lane 3, cold stress (4°C).

Panel B. PhosphorImage of the time course of incorporation of [<sup>35</sup>S]methionine into the 86 kDa heat stress up-regulated protein, immunoprecipitated using the pea mtNDPK antibody. Lane 1, 2 hours; lane 2, 4 hours and lane 3, 8 hours.

Panel C. Western blot of immunoprecipitations of crude mitochondrial proteins prepared from pea leaves exposed to various stresses probed with anti-mtNDPK. Lane 1, control; lane 2, high salt stress (400 mM NaCl); lane 3, oxidative stress (2% H<sub>2</sub>O<sub>2</sub>); lane 4, heat stress (42°C); lane 4, cold stress (4°C).

### Figure 5

Gel filtration of pea mitochondria soluble fraction. Fractions of 1 ml were collected and analysed using the pea mtNDPK antibody. On Y-axis, bottom panel, quantification of the signal corresponding to the pea mtNDPK (analysed with ImageQuant 1.2 software, Molecular Dynamics). At the top, the calibration curve showing the used standards.

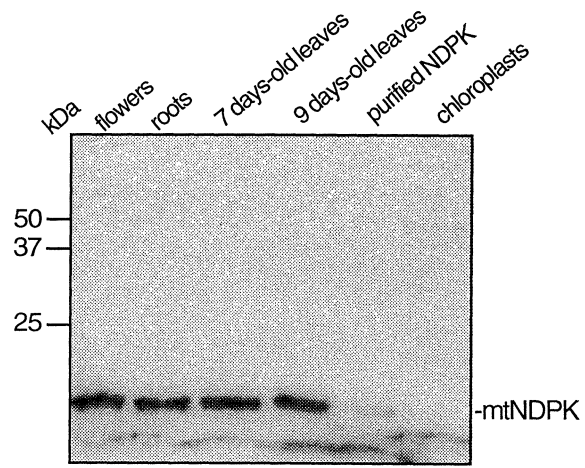
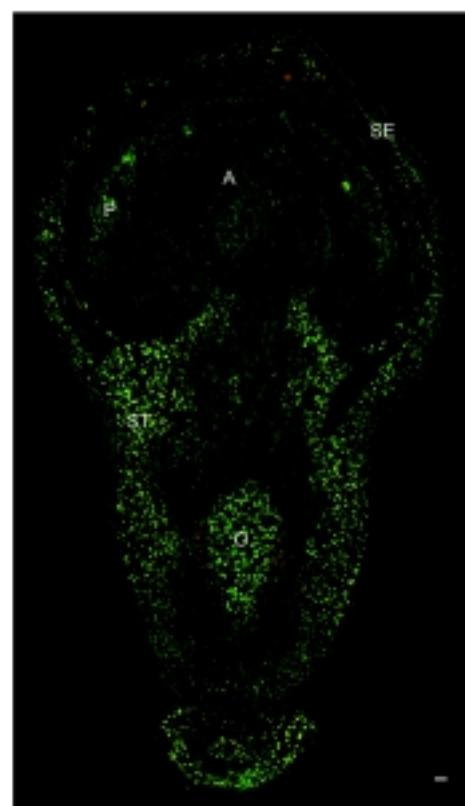
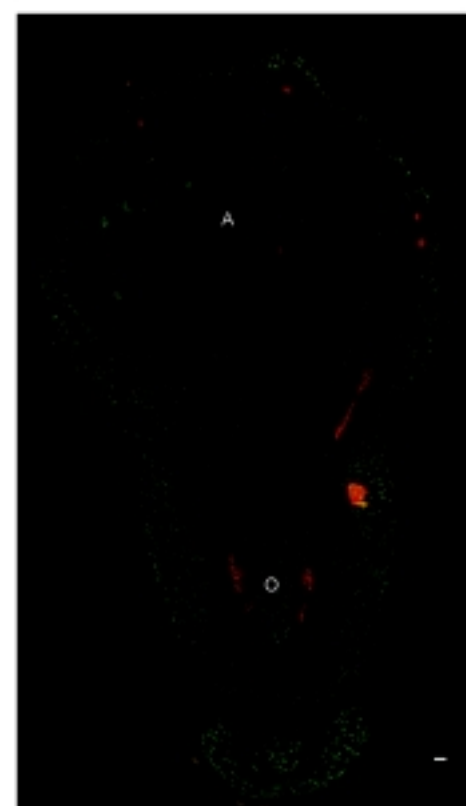


Figure 1

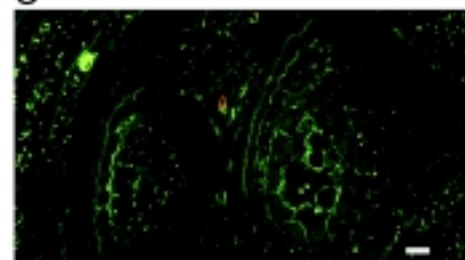
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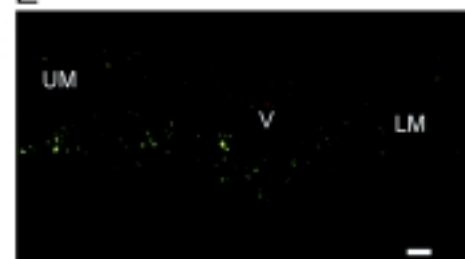
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D



E



F



Figure 2

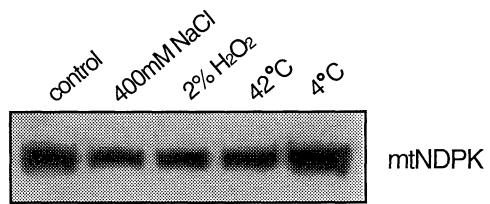


Figure 3

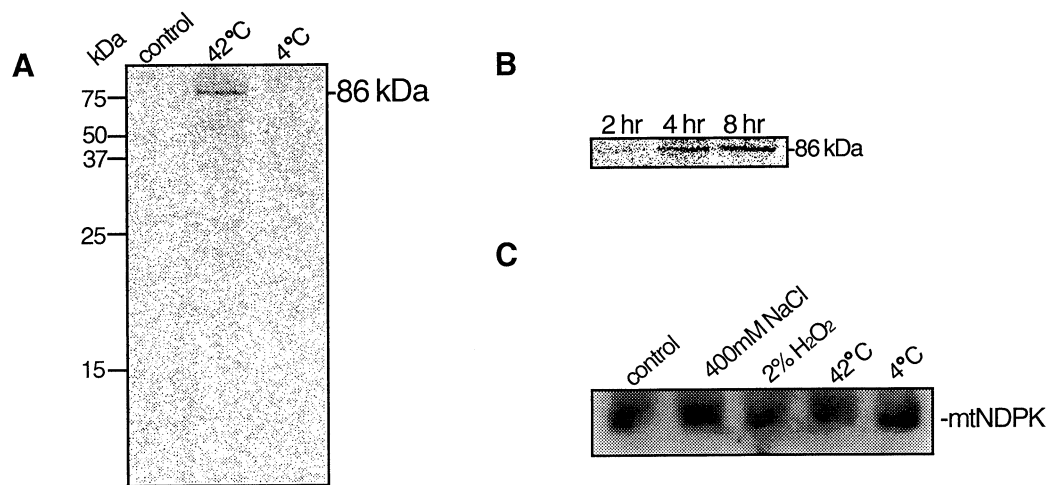


Figure 4

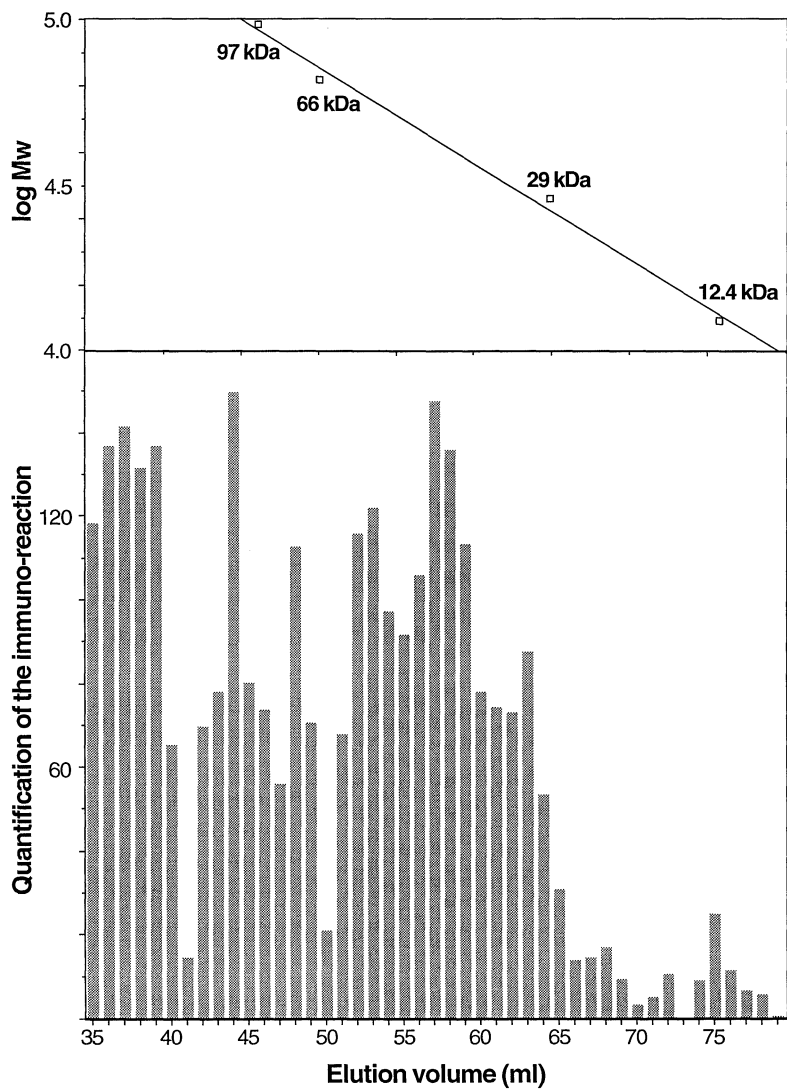


Figure 5

