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## Pyridine Nucleotides in Plant Mitochondria. Amounts, metabolism and contribution to electron transport

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2001

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*Citation for published version (APA):*

Agius, S. C. (2001). *Pyridine Nucleotides in Plant Mitochondria. Amounts, metabolism and contribution to electron transport*. [Doctoral Thesis (compilation), Department of Biology].

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1

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**Pyridine Nucleotides in Plant Mitochondria**  
**Amounts, metabolism and contribution to electron transport**

**Stephanie C. Agius**



**LUND**  
UNIVERSITY

**Department of Plant Physiology**

**Doctoral Dissertation**

**By due permission of the Faculty of Science of Lund University, Sweden, to  
be defended at the Department of Plant Physiology, Sölvegatan 35, Lund, on  
October 5<sup>th</sup>, 2001 at 10 am for the degree of Doctor of Philosophy.**

<b>Organization</b> LUND UNIVERSITY Lund University Department of Plant Physiology Box 117 s-221 00 Lund, Sweden	<b>Document name</b> DOCTORAL DISSERTATION	
	<b>Date of issue</b>	September 2001
	<b>CODEN:</b>	LUNBS/(nbfb-1038)/1- (2001)
<b>Author(s)</b>	Stephanie C. Agius	<b>Sponsoring organization</b>
<b>Title and subtitle</b> Pyridine Nucleotides in Plant Mitochondria Amounts, metabolism and contribution to electron transport		
<b>Abstract</b>  <p>HPLC analysis was used for direct quantification of amounts and redox levels of pyridine nucleotides in plant mitochondria. The amount of NAD(H) and NADP<sup>+</sup> varied, 1.0-3.7 and 0-0.5 nmol (mg protein)<sup>-1</sup>, respectively, depending on the species from which nucleotides were extracted. NADPH was never detected.</p> <p>Submitochondrial localisation of NAD<sup>+</sup>-degrading enzymes indicated that matrix NAD<sup>+</sup> diffuses intact across the inner mitochondrial membrane and is metabolised by NAD<sup>+</sup>-catabolising enzymes in the outer membrane. NAD pyrophosphatase activity was identified as being involved in the pyridine nucleotide degradation.</p> <p>During malate oxidation by isolated <i>Solanum tuberosum</i> mitochondria, the highest levels of NAD(H) reduction were obtained when malic enzyme was active. The lowest NAD(H) reduction levels were seen when conditions favoured malate dehydrogenase activity. Rotenone increased the reduction level of intramitochondrial NADH. Diphenylene iodonium (DPI) inhibited malate oxidation in the presence of rotenone in isolated mitochondria. This result indicated participation of matrix NADPH and the internal rotenone-insensitive, DPI-sensitive, NADPH dehydrogenase during malate oxidation.</p> <p>Antisera raised against peptides deduced from two <i>S. tuberosum</i> genes (<i>nda1</i> and <i>ndb1</i>), homologous to the yeast genes for the internal and external NADH:ubiquinone dehydrogenases specifically cross-reacted with 48 and 61 kDa polypeptides in potato tuber mitochondria, respectively. Native NDA1 and NDB1 occurred as higher molecular mass forms, and show different association to the inner mitochondrial membrane.</p> <p>The physiological significance of pyridine nucleotide turnover and occurrence of multiple NAD(P)H dehydrogenases in the inner mitochondrial membrane is discussed.</p>		
<b>Key words</b> pyridine nucleotides, plant mitochondria, NADPH dehydrogenase, NADH:ubiquinone oxidoreductase, NAD-pyrophosphatase, antibody		
<b>Classification system and/or index terms (if any)</b>		
<b>Supplementary bibliographical information</b>		<b>Language</b> English
<b>ISSN and key title</b>		<b>ISSN</b> 91-7874-143-2
<b>Recipient's notes</b>	<b>Number of pages</b>	<b>Price</b>
	<b>Security classification</b>	

Distribution by (name and address)

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Date 2001-08-27

*The future*

The page just turned  
was yesterday new!  
writing a story of life  
is already past and buried.  
Was truly wonderous  
however,  
in one's own fantasy.  
It is far more wonderous than that,  
the page that today is still unrevealed,  
the page of the future.

*Frieda Aquilina 2001*

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**Populärvetenskaplig sammanfattning (Swedish summary)**

**Acknowledgements**

**References**

**Separate publications**

## Publication List

This thesis is based on the following publications which are referred to in the text by their roman numerals.

- I **Agius S C**, Rasmusson A G, Møller I M (2001) NAD(P) turnover in plant mitochondria. *Aust. J. Plant Physiol.* **28**, 461-470.
- II **Agius S C**, Bykova N V, Igamberdiev A U, Møller I M (1998) The internal rotenone-insensitive NADPH dehydrogenase contributes to malate oxidation by potato tuber and pea leaf mitochondria. *Physiol. Plant.* **104**, 329-336.
- III **Agius S C**, Rasmusson A G, Åkerlund H E, Møller I M (1998) Dynamic changes in the redox level of NAD in potato tuber mitochondria oxidising malate. In *Plant Mitochondria: from Gene to Function*, eds I M Møller, P Gardeström, K Glimelius, E Glaser, pp. 343- 346. Backhuys Publishers, Leiden, The Netherlands.
- IV Rasmusson A G, **Agius S C** (2001) Rotenone-insensitive NAD(P)H dehydrogenases in plants: Immunodetection and distribution of native proteins in mitochondria. *Plant Physiol. Biochem.* In Press.

## Abbreviations

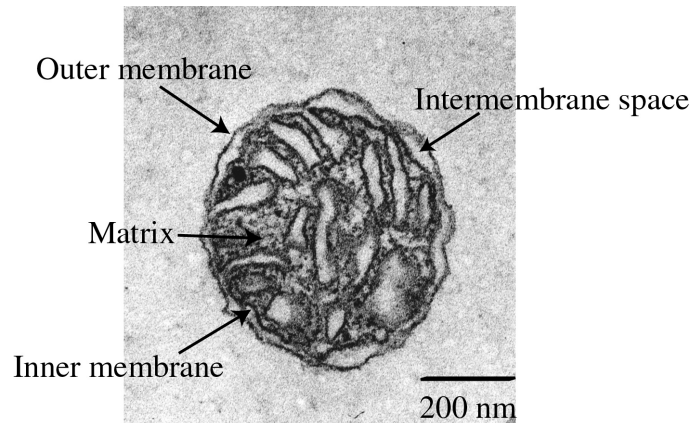
ADP	adenosine diphosphate
ATP	adenosine triphosphate
Ca <sup>2+</sup>	calcium
CMS	cytoplasmic male sterility
DPI	diphenylene iodonium
ETC	electron transport chain
H	hour
HPLC	high performance liquid chromatography
IMS	intermembrane space
MDH	malate dehydrogenase
ME	malic enzyme
NAD(H)	(NAD <sup>+</sup> + NADH)
NADP(H)	(NADP <sup>+</sup> + NADPH)
NAD <sup>+</sup>	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP <sup>+</sup>	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NAD(P)	NAD <sup>+</sup> or NADP <sup>+</sup>
ND <sub>in</sub> (NADH)	matrix facing rotenone-insensitive NADH dehydrogenase
ND <sub>in</sub> (NADPH)	matrix facing rotenone-insensitive NADPH dehydrogenase
NADP <sup>+</sup> -ICDH	NADP <sup>+</sup> -specific isocitrate dehydrogenase
NMN	nicotinamide mononucleotide
OAA	oxaloacetate
SMP	submitochondrial particles
UQ	ubiquinone



## **1 Introduction**

### **1.1 Plant mitochondrial morphology and cellular function**

Mitochondria are small (0.5  $\mu\text{m}$  x 2  $\mu\text{m}$ ), semi-autonomous organelles varying in shape from spherical to filamentous (Figure 1) (Douce, 1985). The number of mitochondria per cell also varies depending on the type and size of the cell and the extent of cellular differentiation (Douce, 1985, McCabe et al., 2000). In general, mitochondria occupy as little as 1% of the total volume of the cell. Mitochondria are principally responsible for energy production, however, their biosynthetic and physiological roles can change depending on cellular demands. For example, mitochondria in plant cells have evolved with chloroplasts, and are integrally involved in processes of ammonium assimilation in plastids, C<sub>4</sub> photosynthesis and photorespiration (Siedow and Day, 2000). In the seeds of certain plants, gluconeogenesis involves the transport of organic acids between the glyoxysome and mitochondrion, facilitating mobilisation of lipid reserves to glucose (Siedow and Day, 2000). Mitochondria can also be instigators of programmed cell death, apoptosis, by release of intermembrane soluble proteins such as cytochrome c through opening of permeability transition pores (Liu et al., 1996, Marzo et al., 1998, Krajewski et al., 1999). This function has been extensively studied in mammals, but evidence has been found for a similar mitochondrial involvement in plants (Balk and Leaver, 2001).



**Figure 1.** Electron micrograph of a mitochondrion in *Solanum tuberosum* .

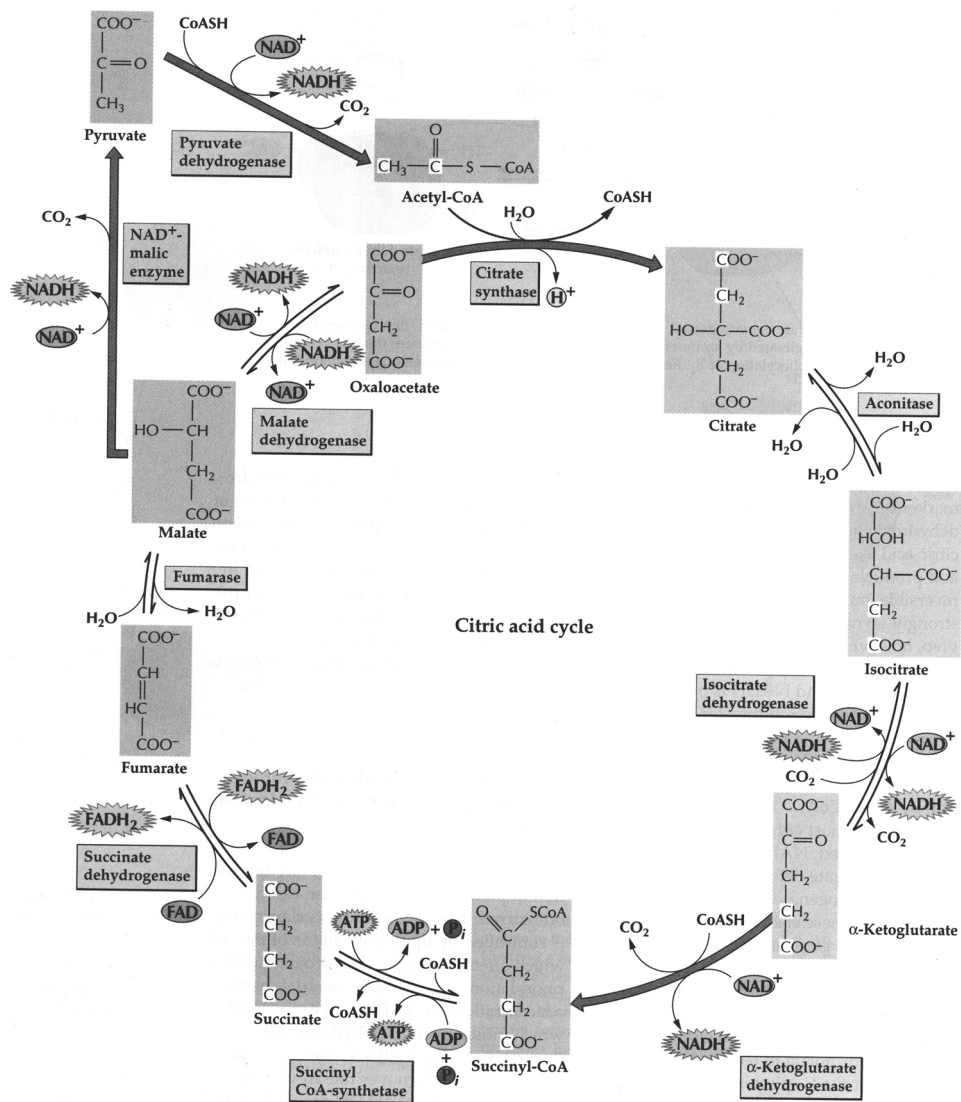
### **1.2 Ultrastructure of plant mitochondria**

Mitochondria possess two membranes, an outer membrane surrounding a highly invaginated inner membrane, which gives rise to structures known as cristae (Figure 1). Enclosed within the inner membrane is the mitochondrial matrix, packed with soluble proteins (0.4 g protein/mL) (Douce, 1985). The compartment between the two mitochondrial membranes is referred to as the intermembrane space (IMS). The metabolite composition of the IMS is considered to be similar to that in the cytosol, due to permeability of the outer membrane, allowing molecules less than 10 kDa to pass through proteinaceous channels (Douce, 1985, Lee et al., 1996). In contrast, the inner membrane is a selectively permeable barrier, preventing charged ions and hydrophilic metabolites from transversing into or out of the mitochondrial matrix.

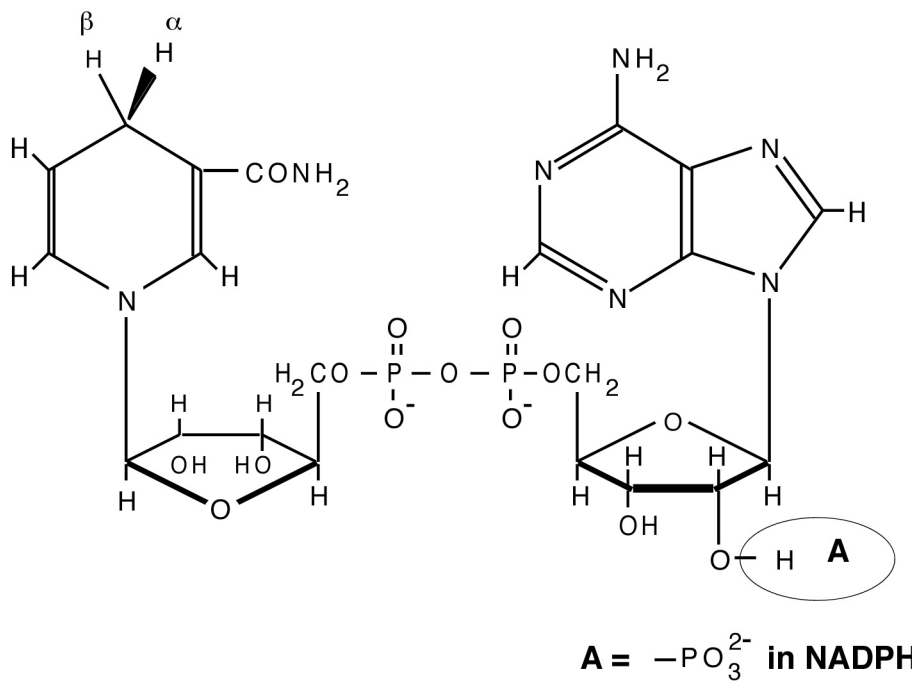
### **1.3 Respiration: glycolysis, citric acid cycle and oxidative phosphorylation**

Respiration can be divided into three phases; Firstly, the conversion of glucose to pyruvate carried out by a series of soluble, cytosolic enzymes which make up the glycolytic pathway. Secondly, the uptake of pyruvate into mitochondria, where pyruvate is oxidatively decarboxylated by the citric acid cycle (Figure 2).

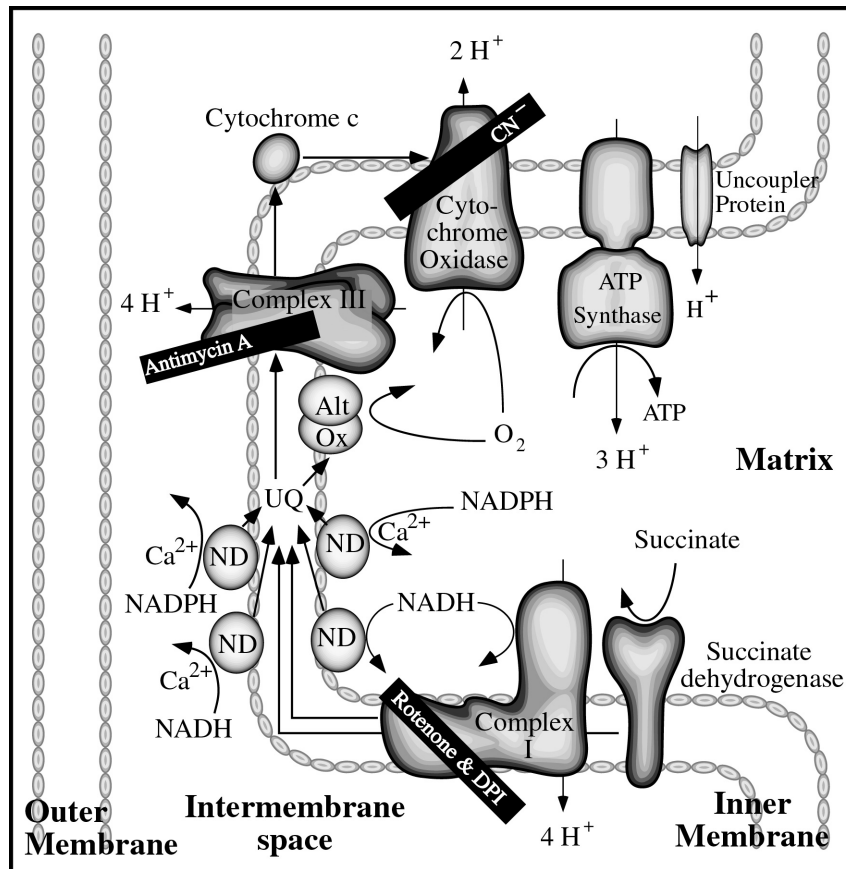
Pyruvate can also be synthesised intramitochondrially by  $\text{NAD}^+$ -malic enzyme ( $\text{NAD}^+$ -ME) from the oxidation of malate. Finally, reducing equivalents (e.g. NADH) (Figure 3), synthesised during glycolysis and the citric acid cycle are oxidised by the mitochondrial electron transport chain (ETC) that ultimately donate electrons to molecular oxygen producing water (Figure 4). The energy released during electron transport is used to transport protons from the matrix to the IMS by complex I, III and IV, the three sites of energy conservation (see below). Proton translocation is used to generate an electrochemical gradient across the inner mitochondrial membrane. Energy stored in this gradient is used for the conversion of ADP to ATP. The coupling of electron transport from substrate oxidation to ATP synthesis is referred to as oxidative phosphorylation.



**Figure 2.** Reactions of the plant citric acid cycle. Pyruvate is completely oxidised to CO<sub>2</sub>. Electrons generated from sequential redox reactions are used to reduce four molecules of NAD<sup>+</sup> to NADH and one molecule of FAD to FADH<sub>2</sub> per pyruvate molecule oxidised. In addition, one molecule of ATP is synthesized by a substrate-level phosphorylation, catalysed by succinyl-CoA synthetase. From Siedow and Day (2000) with permission.



*Figure 3.* Structure of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), reduced species. (Courtesy of A. G. Rasmusson).



**Figure 4.** Organisation of the electron transport chain in the plant mitochondrial inner membrane. Plant mitochondria possess an alternative oxidase (Alt Ox), and up to four alternative rotenone-insensitive NAD(P)H dehydrogenases (ND), in addition to the standard protein complexes 1-IV and ATP synthase common to most eukaryotes. A large pool of ubiquinone (UQ) freely diffusible in the hydrophobic membrane transfers electrons from the dehydrogenases to either complex III or the alternative oxidase. Transmembrane translocation of protons through complexes I, III and IV generates an electrochemical gradient which drives the biosynthesis of ATP, by ATP synthase. Chemical inhibitors are written in white across the respective complexes (Courtesy of A. G. Rasmusson).

The ETC in the inner mitochondrial membrane is composed of four integral multiprotein complexes.

*Complex I* (NADH:ubiquinone oxidoreductase) oxidises matrix-produced NADH and transfers electrons to ubiquinone.

*Complex II* (succinate:ubiquinone oxidoreductase) is the only enzyme in the respiratory chain which is also part of the citric acid cycle. Complex II catalyses the oxidation of succinate, transferring electrons to the ubiquinone pool.

*Complex III* (cytochrome *bc1* complex) oxidises ubiquinol and transfers electrons to cytochrome *c*, a small peripherally attached protein located on the cytoplasmic face of the inner membrane.

*Complex IV* (cytochrome *c* oxidase) oxidises reduced cytochrome *c* and electrons are transferred to molecular oxygen, producing water.

Plant mitochondria possess electron transfer components in the respiratory chain not commonly found in other organisms. There are four additional NAD(P)H dehydrogenases, characterised by their insensitivity towards the complex I inhibitor rotenone (Roberts et al., 1995, Melo et al., 1996), and a quinol oxidase (alternative oxidase) providing an alternative, non-phosphorylating route for the reduction of molecular oxygen to water (Vanlerberghe and McIntosh, 1997).

#### **1.4 Alternative NAD(P)H dehydrogenases in plant mitochondria**

Cytosolic NADH and NADPH is oxidised by two distinct enzymes, in a calcium ( $\text{Ca}^{2+}$ )-dependent manner (Roberts et al., 1995), whereas the matrix facing alternative dehydrogenases oxidise NADH and NADPH in a  $\text{Ca}^{2+}$ -independent and -dependent fashion, respectively (Rasmusson and Møller, 1991, Melo et al., 1996). The presence of rotenone-insensitive NADH dehydrogenases in plant mitochondria is ubiquitous amongst plant species as detected from enzyme activity assays, but the occurrence of alternative internal NADPH dehydrogenases

has only been reported for two plant species (Rasmusson and Møller, 1991, Melo et al., 1996, Paper II).

In *S. tuberosum* *nda1* and *ndb1* genes encode two rotenone-insensitive NADH dehydrogenase homologues orientated towards the internal and external side of the inner membrane, respectively (Rasmusson et al., 1999). In order to be able to detect the presence of alternative NAD(P)H:ubiquinone oxidoreductases in various plant species we have produced antibodies raised against NDA1 and NDB1 proteins (Chapter 4, Paper IV).

The non-proton pumping rotenone-insensitive NAD(P)H dehydrogenases and an alternative oxidase in the plant ETC constitute the non-phosphorylating pathways. The oxidation of substrate by the rotenone-insensitive NAD(P)H dehydrogenases, transfer of electrons to the ubiquinone pool, and oxidation of ubiquinol by alternative oxidase will not contribute to the electrochemical proton gradient and concomitant production of ATP. Instead, energy will be released as heat (Møller, 2001). In animal and plant mitochondria, the uncoupling of substrate oxidation from ATP biosynthesis plays an essential physiological role (Meeuse, 1975, Nicholls and Locke, 1984). Mammals are capable of increasing whole body respiration up to 10 fold, generating heat, due to the presence of an uncoupling protein in the mitochondrial inner membrane facilitating increased proton conductance (Nicholls and Locke, 1984). This is characteristic of homeothermic hibernators, cold-adapted rodents and newborn mammals to prevent shivering (Nicholls and Locke, 1984). An uncoupling protein also occurs in plant mitochondria, and may regulate the energy balance of the cell as has been proposed for the alternative oxidase (Jarmuszkiewicz et al., 1998). In the family Araceae, however, heat produced from activity of alternative oxidase is used to volatilise odorous compounds to attract pollinators (Meeuse, 1975).

Similar to postulated functions of alternative oxidase, the rotenone-insensitive NAD(P)H dehydrogenases may function as non-phosphorylating bypasses when intramitochondrial concentrations of phosphate are low (Rychter et al., 1992), excess substrate is available to the mitochondrial respiratory chain (Svensson and Rasmusson, 2001, Paper II), high membrane potential (Palmer and Møller, 1982),



or impairment of ETC complexes (Day et al., 1976, Rayner and Wiskich, 1983, Cook-Johnson et al., 1999). Stress response of tissue to low temperatures (Fredlund et al., 1991) and growth in the presence of rotenone (Zhang et al., 2001) also affect capacities for rotenone-insensitive NADH oxidation. The physiological role(s) of these enzymes still remains speculative.

NAD(P)H dehydrogenases having different specificities towards their substrate raises the question as to what functional role do NADPH-specific dehydrogenases play in respiration? This is of special interest as their presence challenges the original dogma in biochemical textbooks where NADH was considered to be the sole pyridine nucleotide used during oxidative phosphorylation (Stryer, 1988, Salisbury and Ross, 1992). This question is addressed in more detail in Chapter 3, Papers II and III.

### **1.5 The pyridine nucleotides: NAD(H) and NADP(H)**

Pyridine nucleotides are essential cofactors present within the mitochondrial matrix. They are responsible for continual turnover of the citric acid cycle, providing respiratory substrate for oxidative phosphorylation (Stryer, 1988, Rasmusson and Møller, 1990, Møller and Rasmusson, 1998, Siedow and Day, 2000), scavenging reactive oxygen species (Edwards et al., 1990, Jimenez et al., 1997, Siedow and Day, 2000) and enzyme regulation (e.g. pyruvate dehydrogenase). Additionally, their catabolism provides essential precursors used for synthesis of RNA (Pearson et al., 1993), signalling pathways (e.g. ADP-ribosylation) (reviewed in Ziegler, 2000) and DNA repair (Gaal et al., 1987).

Isolated plant mitochondria have the ability to take up cofactors such as  $\text{NAD}^+$ , and enzymes which are cofactor-dependent (e.g. malate dehydrogenase) and part of a metabolic pathway (e.g. citric acid cycle) can be regulated to some degree by amounts present in the mitochondrial matrix and their redox state (Douce et al., 1997, Papers I, II and III). In *S. tuberosum* mitochondria, a specific carrier present in the inner membrane is responsible for influx of  $\text{NAD}^+$  into the matrix, as well as for efflux (Tobin et al., 1980).  $\text{NAD}^+$  can also be metabolised

by lysed potato tuber mitochondria as reported in a preliminary finding by Pearson and Wilson (1997). Mitochondrial NAD<sup>+</sup>-metabolites may be used for RNA and DNA synthesis as has been demonstrated for chloroplasts (Pearson and Wilson, 1993, Wilson et al., 1996).

NADP<sup>+</sup> is present in plant mitochondria (Harmey, 1966, Ikuma, 1967, Brinkman et al., 1973, Wigge et al., 1993, Papers I, II and III). However, the origin of this cofactor and flux across the inner membrane is unknown. In Chapter 2, Paper I, turnover of NAD(H) and NADP(H) has been studied.

In the following chapters I have investigated amounts, metabolism and contribution of NAD(H) and NADP(H) during electron transport as well as the distribution of NAD(P)H dehydrogenases in plant mitochondria.

## 2 Pyridine nucleotides in plant mitochondria

### 2.1 Amounts of NAD(H) and NADP(H)

Within plant cells the pyridine nucleotides NAD(H) and NADP(H) exist as discrete pools in different subcellular compartments where they carry out numerous functions (section 1.5) (Laloi, 1999, Lisa and Ziegler, 2001).

In plant and animal cells, mitochondria contain a relatively high proportion of the total NAD(H) (Hampp et al., 1985, Wigge et al., 1993, Magni et al., 1999). Following rapid fractionation of barley leaf protoplasts, the amounts of NAD(H) found in mitochondria represented 10-15% of the total cellular NAD(H) pool (Wigge et al., 1993). In potato tuber mitochondria amounts vary from 0.3-3.0 nmol (mg protein)<sup>-1</sup> or 0.3-3.0 mM, assuming a matrix volume of 1  $\mu$ L (mg protein)<sup>-1</sup>) (Harmey, 1966, Brinkman et al., 1973, Tobin et al, 1980, Roberts et al, 1997, Papers I and III). By comparison, the NAD(H) content is higher in mitochondria isolated from metabolically active tissues, such as mung beans and pea leaves (4-6 nmol (mg protein)<sup>-1</sup>) than from potato tubers, a relatively inactive tissue (Ikuma, 1967, Tobin et al., 1980, Lenne et al., 1993).

In general, lower amounts of NADP(H) (0.3-0.7 nmol (mg protein)<sup>-1</sup>) are found in plant mitochondria than NAD(H) (Harmey, 1966, Ikuma, 1967, Brinkman et al., 1973, Wigge et al., 1993, Papers I and III). However, using <sup>31</sup>P-nuclear magnetic resonance, Roberts et al., (1997) found amounts of NADP(H) to be twice as high as NAD(H) in potato tuber mitochondria. In this study, reduced pyridine nucleotides were quantified as breakdown products. Due to the large discrepancy in the literature with respect to the amounts of NADP(H), we chose to independently determine amounts of this coenzyme in potato tuber mitochondria using high performance liquid chromatography (HPLC). HPLC enables direct detection of oxidised and reduced species during an elution run (Paper I).

The amount of NADP(H), 0.2-0.5 nmol (mg protein)<sup>-1</sup> represented only 10-30% of the total mitochondrial pyridine nucleotide pool (Paper I). NADPH was in all cases found to be below the detection limit, even under anaerobiosis where the NAD(H) pool was more than 90% reduced (Paper I). Our results are in agreement with earlier reports (Harmey, 1966, Ikuma, 1967, Brinkman et al., 1973, Wigge et al., 1993, Papers I and III), but disagree with findings of Roberts et al., (1997) concerning the mitochondrial content of NADP(H). Absolute amounts of NADP(H) in the mitochondrial matrix may vary due to the potato variety used for mitochondrial isolation, seasonal variability of tubers, purity of mitochondria and assay conditions prior to nucleotide extraction (Brinkman et al., 1973, Neuburger and Douce, 1983, Paper I).

## 2.2 Matrix NADP(H)-utilising enzymes

NADH is considered to be the major electron carrier catabolised during oxidative phosphorylation, whereas NADPH is primarily used for reductive biosyntheses (e.g. fatty acid synthesis) (Stryer, 1988, Taiz and Zieger, 1998). The matrix of plant mitochondria, however, contains a number of enzymes able to utilise NADP(H), some of which are components of the citric acid cycle, the second phase of respiration (section 1.3) (Rasmusson and Møller, 1990, Møller and Rasmusson, 1998). In addition to the citric acid cycle enzyme NAD<sup>+</sup>-isocitrate dehydrogenase (NAD<sup>+</sup>-ICDH), an NADP<sup>+</sup>-ICDH has been characterised in crude soluble extracts of potato tuber mitochondria (Rasmusson and Møller, 1990). Interestingly, the NADP<sup>+</sup>-ICDH has a lower K<sub>m</sub> for isocitrate than NAD<sup>+</sup>-ICDH (Rasmusson and Møller, 1990, Chen and Gadal, 1990, Gálvez and Gadal, 1995). Therefore, at low isocitrate concentrations in the mitochondrial matrix, NADP<sup>+</sup>-ICDH will contribute to isocitrate oxidation to a greater degree than NAD<sup>+</sup>-ICDH, provided the NADP<sup>+</sup> concentration is sufficient to support this oxidation (Rasmusson and Møller, 1990). Malic enzyme, also part of the plant citric acid cycle can reduce both NAD<sup>+</sup> and NADP<sup>+</sup>, but prefers NAD<sup>+</sup> *in vitro* (Macrae,

1971, Grover et al., 1981). Enzymes not part of the citric acid cycle but involved in the conversion of proline to glutamate,  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenase (Forlani et al., 1997), ammonium assimilation, glutamate dehydrogenase (Turano et al., 1996), and synthesis of folate and thymidylate, methylenetetrahydrofolate dehydrogenase (Neuburger et al., 1996) all reduce matrix  $\text{NADP}^+$ .

Another  $\text{NADP(H)}$ -utilising enzyme, the energy-linked transhydrogenase, reduces  $\text{NADP}^+$  by  $\text{NADH}$ , driven by translocation of a proton into the matrix (Hoek and Rydström, 1988). This enzyme found in mammalian mitochondria maintains  $\text{NADP(H)}$  in a more reduced state than  $\text{NAD(H)}$ . In plant mitochondria complex I, and another inner mitochondrial membrane enzyme display transhydrogenase activity (Bykova et al., 1999). However, considering the low reduction level of  $\text{NADP(H)}$  in plant mitochondria (Paper I), the roles of these transhydrogenases are likely to differ from mammalian transhydrogenase activity.

### **2.3 Cofactor uptake and source of mitochondrial $\text{NAD}^+$ and $\text{NADP}^+$**

The inner mitochondrial membrane is an impermeable barrier to the negatively charged cofactors  $\text{NAD}^+$  and  $\text{NADP}^+$  (Douce, 1985). The mechanism by which added  $\text{NAD}^+$  can stimulate  $\text{NAD}^+$ -linked substrate oxidation and matrix  $\text{NADH}$  dehydrogenase activity is therefore unclear. Using radiolabelled ( $^{14}\text{C}$ )  $\text{NAD}^+$ , Tobin et al., (1980) were able to demonstrate the presence of a transport mechanism for net uptake of  $\text{NAD}^+$ . The rate of  $\text{NAD}^+$  accumulation into the matrix followed Michaelis-Menton kinetics (Neuburger and Douce, 1983). Elevated levels of matrix  $\text{NAD}^+$ , reduced by  $\text{NAD}^+$ -linked dehydrogenases (e.g. MDH) and reoxidised by  $\text{NADH}$  dehydrogenases (e.g.  $\text{ND}_{\text{in}}(\text{NADH})$ ) could explain the increased respiratory rate of  $\text{NAD}^+$ -linked substrate oxidation (Palmer and Møller, 1982).

NAD<sup>+</sup> uptake could be inhibited by uncouplers, indicating NAD<sup>+</sup> influx is an energy-dependent event (Neuburger et al., 1985). In comparison, mitochondria incubated in a medium lacking NAD<sup>+</sup> and a respiratory substrate progressively lose NAD<sup>+</sup> in a temperature-dependent manner. The loss was proposed to be via the NAD<sup>+</sup> carrier (Neuburger et al., 1985).

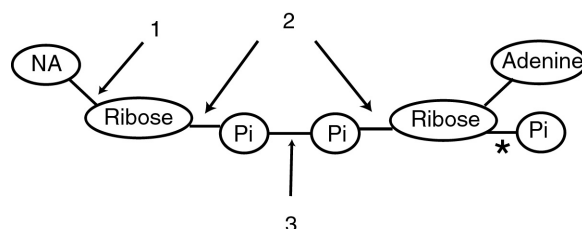
In plant mitochondria the presence of a specific NAD<sup>+</sup> carrier allows for the potential exchange of this coenzyme between mitochondria and the surrounding cytosol *in vivo*. By comparison, the source of matrix NADP<sup>+</sup> is uncertain (Møller, 2001). Rasmusson and Møller (1990) reported that externally added NADP<sup>+</sup> stimulated the oxidation of NAD(P)<sup>+</sup>-linked substrates in potato tuber mitochondria, demonstrating the uptake of NADP<sup>+</sup> into the matrix. However, when this experiment was repeated using NAD<sup>+</sup>-free NADP<sup>+</sup> no stimulation was observed (Agius S C, Rasmusson A G and Møller I M, unpublished). Therefore, this stimulatory effect from added NADP<sup>+</sup> may have come from contaminating NAD<sup>+</sup> in the commercial preparation of NADP<sup>+</sup> (Møller and Rasmusson, 1998). NADP<sup>+</sup> uptake from NAD<sup>+</sup>-free NADP<sup>+</sup> has however been reported in pea leaf mitochondria (Bykova and Møller, 2001).

## **2.4 Biosynthesis and metabolism of NAD<sup>+</sup> and NADP<sup>+</sup>**

If certain species of plant mitochondria are impermeable to NADP<sup>+</sup>, its synthesis should occur *in situ*. NAD<sup>+</sup> kinase activity has been reported in plant mitochondria, where the enzyme was localised to the outer membrane, thus unable to synthesize matrix NADP<sup>+</sup> (Dieter and Marmé, 1984, Møller, 2001). A mitochondrial NAD<sup>+</sup> kinase found in the matrix has not been reported, but it is a likely source of NADP<sup>+</sup> in plant mitochondria.

Metabolism and resynthesis of NAD<sup>+</sup> is referred to as the pyridine nucleotide cycle (Wagner et al., 1986). Regeneration of NAD(P)<sup>+</sup> is necessary to

compensate for  $\text{NAD(P)}^+$ -consuming reactions (Lisa and Ziegler, 2001, Hagen and Ziegler, 1997). In rat liver homogenates, three  $\text{NAD}^+$ -catabolising activities have been identified (Figure 5); NAD pyrophosphatase, responsible for the cleavage of pyrophosphate bonds yielding AMP and NMN. Phosphodiesterase which cleaves at the phosphate group producing adenosine, nicotinamide-ribose and pyrophosphate, and NAD glycohydrolase yielding nicotinamide and ADP-ribose by breakage of the glycosidic bond (Hagen and Ziegler, 1997). Similarly, in partially purified enzyme extracts from tobacco and tomato roots, enzyme activities involved in the recycling of  $\text{NAD}^+$  have been detected (Wagner et al., 1985 and 1986).



**Figure 5.** Enzymatic cleavage of  $\text{NAD}^+$ . (1) NAD glycohydrolase produces nicotinamide and ADP-ribose. (2) phosphodiesterase produces adenosine, nicotinamide ribose and pyrophosphate. (3) NAD pyrophosphatase cleaves the diphosphate bond yielding AMP and NMN. The 2'-phosphate denoted by the asterisk is present in  $\text{NADP}^+$ , not in  $\text{NAD}^+$ . Figure adapted from Hagen and Ziegler (1997).

In mammalian mitochondria an  $\text{NAD}^+$  glycohydrolase has been localised to the outer membrane (Boyer et al., 1993). Although, in plant mitochondria, a preliminary report indicated  $\text{NAD}^+$  breakdown (Pearson and Wilson, 1997, no  $\text{NAD}^+$ -catabolising enzyme activities were detected. Thus, it was uncertain whether  $\text{NAD}^+$  degradation and/or synthesis in plants takes place in mitochondria or in the cytoplasm.

Neuburger and Douce (1983) demonstrated a decline of intramitochondrial  $\text{NAD}^+$  in potato tuber mitochondria during incubation on ice and mentioned that the majority of  $\text{NAD}^+$  could be recovered in the surrounding medium if the pH was maintained below 7.0. To investigate the fate of this lost  $\text{NAD}^+$  we incubated

mitochondria at 0°C and pH 7.2 for a period of 48 h, conditions previously shown to decrease matrix NAD<sup>+</sup> content by 90% (Neuburger and Douce, 1983). Following centrifugation, the mitochondrial pellet and surrounding medium were analysed for NAD<sup>+</sup> metabolites (Paper I).

Intramitochondrial NAD<sup>+</sup> declined with time, but no NAD<sup>+</sup> was recovered in the surrounding medium (Paper I). Two NAD<sup>+</sup> metabolites, AMP and NMN were detected in significant amounts, and adenosine to a lesser extent. In the mitochondrial pellet, the amount of AMP increased during the first 24 h then steadily declined. In the supernatant, NMN increased linearly with time. The presence of AMP, NMN and adenosine indicates that NAD<sup>+</sup> was metabolised by an NAD-pyrophosphatase and a phosphodiesterase (Figure 5).

Although the temperature-dependent loss of NAD<sup>+</sup> from the mitochondrial matrix has been well established (Neuburger and Douce, 1983, Neuburger et al., 1985, Lenne et al., 1993, Paper I), the loss of NADP<sup>+</sup> has never been investigated. We chose to monitor the potential loss of NADP<sup>+</sup> over time. In contrast to the loss of NAD<sup>+</sup> and synthesis of NAD<sup>+</sup>-metabolites with time, no significant loss of NADP<sup>+</sup> could be detected.

Analysis of metabolites from long-term incubation of plant mitochondria clearly indicated degradation of NAD<sup>+</sup>. However, the presence of metabolites in the pellet and surrounding medium was unable to clarify the precise location of NAD<sup>+</sup>-catabolising enzymes. Therefore mitochondria were subfractionated, incubated with NAD<sup>+</sup> or NADP<sup>+</sup>, and the NAD(P)<sup>+</sup>-metabolites analysed. NAD<sup>+</sup> pyrophosphatase and phosphodiesterase activity were localised to the outer membrane fraction (Paper I). It was not possible to establish NAD<sup>+</sup> glycohydrolase activity due to the presence of ADP-ribose in control nucleotide extractions.

NADP<sup>+</sup> was also metabolised by the outer membrane fraction, however, activities were 5-10 times lower than those observed with NAD<sup>+</sup> (Paper I). This



indicates that  $\text{NAD}^+$ -catabolising enzymes may not be completely specific for their substrate. Interestingly, adenosine was detected after a 60 min incubation of outer membrane with  $\text{NADP}^+$ . Its presence indicates that if phosphodiesterase is the responsible enzyme initiating cleavage of  $\text{NADP}^+$ , a phosphatase must hydrolyse the 2' phosphate to produce adenosine (Figure 5) (Paper I).

In isolated plant mitochondria, we conclude that  $\text{NAD}^+$  is transported intact across the inner membrane, and is metabolised by  $\text{NAD}^+$ -catabolising enzymes in the outer membrane. Neuburger et al., (1985) previously demonstrated that addition of N-4-azido-2-nitrophenyl-4-aminobutyryl- $\text{NAD}^+$  prevented the efflux of  $\text{NAD}^+$  from intact mitochondria. The localisation of these  $\text{NAD}^+$ -degrading enzymes to the outer membrane supports this observation. However, to which side of the outer membrane  $\text{NAD}^+$ -catabolising enzymes associate is not known. Following breakdown of  $\text{NAD}^+$ , soluble  $\text{NAD}^+$  metabolites could be transported to the mitochondrial matrix. This could partly explain the presence of AMP and adenosine in mitochondria following long-term storage on ice (Paper I).

### 3 Redox levels of NAD(H) and NADP(H) during respiration

#### 3.1 Mitochondrial respiratory states

Pyridine nucleotides occur in an oxidised or reduced state. The redox level of NAD(H) and NADP(H) can be manipulated *in vitro* by the respiratory state of the mitochondria (Ikuma, 1967, Tobin et al., 1980, Neuburger et al., 1984, Wigge et al., 1993, Papers I and III), referred to as state 1-5 (Chance and Williams, 1956). In polarographic assays, isolated mitochondria added to an oxygenated medium oxidise endogenous substrates very slowly (state 1). The addition of respiratory substrate increases the rate of oxygen consumption (state 2), but it is limited by a high membrane potential. The addition of ADP facilitates higher rates of respiration by decreasing the membrane potential via ATP synthesis (state 3). The conversion of all available ADP to ATP will decrease the respiratory rate, as the membrane potential rises again (state 4). Oxidation of respiratory substrate will be completely stopped when mitochondria enter anoxic conditions (state 5).

The addition of malate to isolated plant mitochondria caused a reduction of the matrix pyridine nucleotide pool (state 2) (Neuburger et al., 1984, Papers I and III). The addition of ADP increased oxygen consumption, with the concomitant oxidation of matrix NADH (state 3), followed by a re-reduction of matrix NAD<sup>+</sup> during state 4 conditions (Neuburger et al., 1984). These steady state changes of intramitochondrial pyridine nucleotides, monitored using fluorescence spectroscopy, were indirect measurements of the nucleotide's redox state. Fluorescence cannot discriminate between NAD(H) and NADP(H), and considering the high amounts of NADP(H) reported by Roberts et al., (1997), it was therefore uncertain to what extent the two coenzymes contributed to produce this fluorescence signal (Neuburger et al., 1984, Papers I and III). In addition, due to different fluorescence yields from free and bound NAD(H), detection is only semiquantitative, and metabolically inactive nucleotide pools will not be detected (Paul and Schneckenburger, 1996, Paper I).

In our investigation, dynamic changes in the redox level of NAD(H) were observed during malate oxidation in isolated mitochondria (Papers I and III). By comparison, the amount of NADPH was below the detection limit ( $0.1 \text{ nmol (mg protein)}^{-1}$ ), under all physiological states. This prevented us from determining to what extent the redox state of NADP(H) follows that of NAD(H) during oxidation of malate. High levels of NADP(H) reduction in potato tuber mitochondria under state 1 conditions have been reported (Brinkman et al., 1973). Our results, obtained using direct determination of intramitochondrial NAD(H) and NADP(H), confirm results obtained by fluorescence signals, previously assumed to primarily reflect the redox state of NAD(H), and describe redox changes quantitatively (Neuburger et al., 1984). We observed the highest levels of NAD(H) reduction under anaerobiosis in potato tuber mitochondria oxidising malate (Paper I and III). Similarly, pea leaf mitochondria exposed to anaerobiosis in the presence of respiratory substrate, increased the reduction level of NAD(H) as determined by enzymatic cycling following extraction (Wigge et al., 1993). Lower NAD(H) reduction levels were observed in pea leaf (Wigge et al., 1993) and potato tuber mitochondria (Papers I and III) under state 3 conditions, as compared to state 4, consistent with previous results (Neuburger and Douce, 1983, Neuburger et al., 1984).

We have demonstrated that reduction levels of intramitochondrial pyridine nucleotides range from completely oxidised to fully reduced during malate oxidation. Therefore the entire pool of NAD(H) can be metabolically active during oxidation of a respiratory substrate (Papers I and III). These changes follow the same pattern when observed using fluorescence spectroscopy (Ikuma, 1967, Tobin et al., 1980, Neuburger and Douce, 1983, Neuburger et al., 1984), enzymatic cycling (Wigge et al., 1993) or HPLC measurement (Paper I & III), indicating that free and bound NAD(H) behave similarly (Tobin et al., 1980, Møller and Palmer, 1982, Palmer et al., 1982, Møller and Lin, 1986).

### 3.2 The redox state of NAD(H) during malate oxidation

During malate oxidation not only the respiratory state of the mitochondria can alter the redox state of the pyridine nucleotide pool, but also the enzyme responsible for reduction of  $\text{NAD}^+$  (Tobin et al., 1980, Neuburger et al., 1984). In isolated mitochondria, malate dehydrogenase (MDH) catalyses the oxidation of malate to oxaloacetate (OAA), with the concomitant reduction of  $\text{NAD}^+$ . The NADH produced is rapidly oxidised by internal NADH dehydrogenases of the ETC, and OAA is excreted from the mitochondrial matrix (Walker and Oliver, 1983). This product removal allows for continued oxidation of malate, until OAA accumulates in the matrix, decreasing the equilibrium concentration of NADH, and rate of malate oxidation (Palmer et al, 1982, Neuburger et al, 1984). This inhibition however, can be circumvented by the participation of  $\text{NAD}^+$  malic enzyme ( $\text{NAD}^+$ -ME).  $\text{NAD}^+$ -ME also oxidises malate, but differs from MDH in that it is able to provide a constant source of NADH, despite equilibrium conditions of MDH (Palmer and Møller, 1982). Therefore,  $\text{NAD}^+$ -ME increases the intramitochondrial concentration of NADH, which in turn can be used to reduce previously accumulated OAA. When all excess OAA has been removed, NADH is once again available to the respiratory NADH dehydrogenases.

By manipulating the external pH of the assay medium, we could selectively activate  $\text{NAD}^+$ -ME and MDH (Macrae, 1971, Tobin et al., 1980, Wiskich and Day, 1982), quantifying amounts and redox levels of mitochondrial pyridine nucleotides. The reduction level of NAD(H) was investigated under three different conditions; (1) at pH 6.5, where the major product of malate oxidation is pyruvate, thus activity is due to ME, (2) at pH 7.5, where ME is inactive (Tobin et al., 1980), favouring MDH activity and, (3) at pH 7.2, where both MDH and ME are active (Macrae, 1971, Tobin et al., 1980, Wiskich and Day, 1982, Papers I and III). In general, higher rates of malate oxidation and levels of NAD(H) reduction were obtained under conditions favouring the activity of ME. By comparison, under all respiratory states, the lowest rates of malate oxidation and NAD(H)

reduction levels were seen when MDH was active (Papers I and III). No NADH was detected under state 3 conditions, at pH 7.5, even in the presence of rotenone, which has previously been shown to increase the reduction level of NAD(H) (Tobin et al., 1980, Palmer et al., 1982, Neuburger et al., 1984). In our investigation, the addition of rotenone increased the reduction level of NAD(H), above that of state 3, in mitochondria oxidising malate at low pH, favouring the involvement of ME (Papers I and III).

### **3.3 Matrix-facing NAD(P)H dehydrogenases in the electron transport chain**

NADH synthesised during malate oxidation can be oxidised by two matrix-facing NADH:ubiquinone oxidoreductases; the rotenone-sensitive complex I with a low  $K_m(\text{NADH})$ , and the rotenone-insensitive NADH dehydrogenase  $\text{ND}_{\text{in}}(\text{NADH})$  which has a 10-fold higher  $K_m(\text{NADH})$  (Møller and Palmer, 1982). During operation of MDH, intramitochondrial NADH levels are not high enough to engage  $\text{ND}_{\text{in}}(\text{NADH})$  (Wiskich and Day, 1982, Palmer and Møller, 1982). Instead oxidation of NADH is solely by complex I. By comparison,  $\text{NAD}^+$ -ME increases NADH levels, and participation of the rotenone-insensitive bypass (Neuburger et al., 1984, Paper II). However, is the rotenone-insensitive NADPH dehydrogenase ( $\text{ND}_{\text{in}}(\text{NADPH})$ ) also contributing to this bypass, and if so, is the activity of  $\text{ND}_{\text{in}}(\text{NADPH})$  dependent on the redox level of NADH during malate oxidation?

### **3.4 The redox level of NAD(H) and participation of the rotenone-insensitive NADPH dehydrogenase**

Although matrix NADPH has never previously been shown to be oxidised by the ETC in intact mitochondria, the sensitivity of  $\text{ND}_{\text{in}}(\text{NADPH})$  towards the inhibitor DPI (Melo et al., 1996), demonstrated using SMP, made it possible to

determine the contribution of this dehydrogenase during oxidation of citric acid cycle substrates (Paper II).

The sensitivity of the three internal NAD(P)H:ubiquinone oxidoreductases towards DPI was investigated in SMP (Paper II). DPI was found to inhibit complex I, ND<sub>in</sub>(NADPH) and ND<sub>in</sub>(NADH) with a K<sub>i</sub> of 3.7, 0.17 and 63 μM respectively, and the 400 fold difference in K<sub>i</sub> between the two rotenone-insensitive NAD(P)H dehydrogenases made possible the use of DPI inhibition to estimate ND<sub>in</sub>(NADPH) contribution to malate oxidation by intact mitochondria. The oxidation of malate in the presence of rotenone, to inhibit complex I activity, was further inhibited by DPI (Paper II). This result suggests binding of DPI to the ND<sub>in</sub>NADPH. We conclude that the NADPH rotenone-insensitive dehydrogenase contributes to malate oxidation *in vitro*.

The contribution of ND<sub>in</sub>(NADPH) depended on the redox level of pyridine nucleotides. Cofactor levels were manipulated in mitochondria by allowing malate oxidation to occur at pH 6.8 (favouring NAD<sup>+</sup>-ME activity), and 7.2 (favouring MDH activity), in the presence or absence of an OAA-removal system (section 3.2) (Paper II). In pea leaf mitochondria, DPI inhibited malate oxidation in the presence of rotenone under conditions favouring high levels of NAD(H) reduction (pH 6.8), whereas no inhibition was seen when levels of pyridine nucleotides are typically low (pH 7.2) (Papers I and III). High levels of pyridine nucleotide reduction have previously been shown to engage the rotenone-insensitive NADH dehydrogenases (Møller and Lin, 1986, Neuburger et al., 1984). In this study, MDH from pea leaf mitochondria was unable to reduce the cofactor NADP<sup>+</sup>, unlike ME (section 2.2). This may explain the lack of ND<sub>in</sub>(NADPH) activity during malate oxidation at pH 7.2 in pea leaf mitochondria, presuming that NAD<sup>+</sup>-ME was not activated under these conditions producing NADPH (Paper II).

The amount of NADP(H) (0.1-0.2 nmol (mg protein)<sup>-1</sup>) in plant mitochondria is approximately 10 times lower than NAD(H) (Papers I and III). Assuming the NADP(H) pool is completely reduced, this amount, equivalent to a matrix

concentration of 0.1-0.2 mM, is sufficient to engage the ND<sub>in</sub>(NADPH) to 80% of its capacity (Paper II).

We have established the participation of ND<sub>in</sub>(NADPH) activity by inhibiting malate oxidation with DPI in the presence of rotenone. Although the concentration of DPI used (5 μM) was chosen to inhibit only ND<sub>in</sub>(NADPH), K<sub>i</sub> 0.17 μM (Paper II), the matrix concentration of this inhibitor may be higher in actively respiring mitochondria (Møller, 2001). DPI, a positively charged molecule with a delocalised charge permeates lipid bilayers (Roberts et al., 1995, Melo et al., 1996, Paper II). Due to the large membrane potential across the inner membrane (-180 mV (inside negative)), DPI may accumulate in the matrix, partially inhibiting ND<sub>in</sub>(NADH) (Møller, 2001). In addition, it was not possible to determine to what extent ND<sub>in</sub>(NADPH) participates in respiration when complex I is active (Møller, 2001). It is however unlikely that complex I will compete with ND<sub>in</sub>(NADPH) for substrate, as the K<sub>m</sub>(NADPH) for this dehydrogenase is 40 times higher than the K<sub>m</sub> of ND<sub>in</sub>(NADPH) (Rasmusson and Møller, 1991).

## **4 Membrane association and immunodetection of alternative NAD(P)H dehydrogenases in mitochondria**

### **4.1 Import and membrane association of internal NAD(P)H dehydrogenases**

The NADH:ubiquinone oxidoreductases can be divided into three groups; (Group 1) enzymes which couple electron transfer from NADH to ubiquinone (UQ), to proton translocation across the inner mitochondrial membrane (complex I), (Group 2) alternative, non-proton pumping NADH dehydrogenases (Yagi, 1991, Yagi et al., 1998), and (Group 3) Na<sup>+</sup>-translocating NADH:UQ oxidoreductases, restricted to bacteria (Nakayama et al., 1998).

Group 2 NADH dehydrogenases characterised in bacteria, yeast and plants are in general single polypeptides with an apparent molecular mass of 50-60 kDa. They contain FAD as the sole prosthetic group as compared to complex I which possesses FMN and several iron-sulphur centres as prosthetic groups (Rasmusson et al., 1999, Kersher, 2000, Joseph-Horne et al., 2001, Møller 2001).

Mitochondria of *Saccharomyces cerevisiae* lack complex I activity, but can oxidise intramitochondrial NADH by an internal, rotenone-insensitive dehydrogenase (NDI1), which has been purified and characterised *in vitro* (de Vries and Grivell, 1988, de Vries et al., 1992). NDI1 encodes a single polypeptide synthesised *in vitro* as a precursor with a molecular mass of 53 kDa for the mature protein (de Vries and Grivell, 1988).

Processing of a precursor protein was also seen with import of a plant mitochondrial protein, NDA1, homologous to NADH dehydrogenases of bacteria and yeast, suggested to be an internal rotenone-insensitive NADH dehydrogenase (Rasmusson et al., 1999). Addition of the cross-linker, 3,3'-dithiobis-sulfosuccinimidylpropionate (DTSSP) did not cross-link NDA1 to the external side of the inner membrane, indicating that NDA1 does not span the inner mitochondrial membrane. No obvious transmembrane segments were detected in hydropathy plots. It is therefore unlikely that NDA1 is an integral transmembrane protein, but rather is peripherally attached to the inner membrane by the C



terminus (Rasmusson et al., 1999). Alternatively, NDA1 may expose a short segment to the external, cytoplasmic-facing surface of the inner membrane (Rasmusson et al., 1999).

In plant mitochondria, there have been several attempts to purify the rotenone-insensitive NAD(P)H dehydrogenases (Rasmusson et al., 1993, Luethy et al., 1995, Menz and Day, 1996a and b). However, different methodologies used for purification and species, make biochemical comparison of the proteins difficult. No clear identification was reached regarding plant mitochondrial alternative NAD(P)H dehydrogenases (Møller, 1997, Møller and Rasmusson, 1998). Therefore a specific and reliable assay is needed for detection of the respective proteins (Paper IV).

#### **4.2 Immunodetection of the internal rotenone-insensitive NADH dehydrogenase**

NDA antiserum was raised against a peptide deduced from potato *nda1*, a homologue to yeast and bacterial genes encoding alternative NADH dehydrogenases. The peptide used was a relatively unconserved segment of the NDA1 polypeptide, immediately upstream of the second nucleotide binding motif (Rasmusson et al., 1999, Paper IV). To investigate the specificity of the antiserum, cDNA coding for NDA1 of *S. tuberosum* was expressed as a hybrid protein in *Escherichia coli* with an N-terminal S-tag (Rasmusson et al., 1999). NDA antiserum specifically recognised NDA1 hybrid protein in *E. coli* (Paper IV).

NDA antibodies were used for specific detection of mitochondrial NDA1 in different species, determining native size of the protein and investigating membrane association (Paper IV).

Using antiserum raised against NDA1 we could specifically detect a 48 kDa polypeptide in potato tuber mitochondria (Paper IV), consistent with the theoretical molecular mass of NDA1 (Rasmusson et al., 1999). NDA1 may reside in the inner membrane as a tri- or tetrameric structure as indicated from the native

molecular mass (150-200 kDa) (Paper IV). No cross reactivity of NDA1 with chloroplasts and a microsomal fraction, indicated organellar specificity (Paper IV). A broad diffuse band seen in peroxisomes may be due to catalase, a predominant protein in these organelles (Struglics et al., 1993) with similar molecular mass to NDA1 (Paper IV).

NDA antiserum recognised NDA1 in mitochondria isolated from flowers, leaves and tubers, however, the intensity of the signal varied. The signal intensity was more pronounced in leaf mitochondria than flowers or tubers (Paper IV). By comparison, other immunodetected respiratory chain components (complex I subunits) show almost no variation in the signal intensity in the respective tissues (Paper IV). *nda1* is light regulated, and the protein is suggested to have a specific role in photorespiration (Svensson and Rasmusson, 2001). As the leaf tissue is the primary organ for photosynthesis in *S. tuberosum*, one would expect a high expression of this protein as compared to non-photosynthetic tissues. However, as different isoenzymes may be present in different tissues, a quantitative comparison is difficult to make.

Although NDA antiserum cross reacted specifically with mitochondrial polypeptides from various plant sources, no signal was detected in mitochondria isolated from *Ipomea batatas* and *Zea mays* (Paper IV). The absence of an immunoreactive signal suggests the peptide sequence of the epitope used for production of NDA antibody is not conserved in these species.

Use was made of NDA1 antiserum to investigate mitochondrial membrane association. The NDA1 signal was found to sediment with the inner membrane following sonication of potato tuber mitochondria or treatment with digitonin (Paper IV). In contrast, Triton X-100 efficiently solubilised NDA1. Similarly, detergent was necessary for solubilisation of hybrid NDA1 protein from bacterial membranes (Rasmusson et al., 1999). We conclude that NDA1 is firmly bound to the inner membrane of plant mitochondria (Paper IV).

### 4.3 Import and membrane association of external NAD(P)H dehydrogenases

In *S. cerevisiae*, two genes *nde1* and *nde2* encode mitochondrial NADH dehydrogenases catalysing the oxidation of cytosolic NADH (Luttik et al., 1998). By comparison, *Yarrowia lipolytica*, the obligate aerobic yeast encodes only one external alternative NADH:UQ oxidoreductase (Kersher et al., 1999).

In plant mitochondria, a gene encoding an externally facing rotenone-insensitive dehydrogenase (*ndb1*) has been cloned (Rasmusson et al., 1999). *In vitro* import of NDB1 into mitochondria did not result in processing. Perhaps NDB1 lacks a cleavable presequence as has been found in some outer membrane proteins (Freeman et al., 1997). By comparison, in *Neurospora crassa*, a cDNA clone encoding for a 64 kDa, external, Ca<sup>2+</sup>-dependent, NADPH dehydrogenase is synthesised *in vitro* as a larger precursor, indicating removal of presequence (Melo et al., 2001).

Similar to NDE1 of *N. crassa* (Melo et al., 2001), potato NDB1 protein contains an insertion, related to Ca<sup>2+</sup>-binding EF-hand motifs (Rasmusson et al., 1999). It has previously been speculated that Ca<sup>2+</sup>-binding may induce a conformational change in Ca<sup>2+</sup>-dependent, external NAD(P)H dehydrogenases, facilitating association to the inner mitochondrial membrane (Møller, 1997). Following import of hybrid NDB1, DTSSP cross-linked the protein suggesting NDB1 is exposed to the intermembrane space. Therefore, Rasmusson et al., (1999) suggested that the NDB1 protein represents the external NADH dehydrogenase in plant mitochondria. In addition, the NDB1 hybrid was released as a soluble protein in the absence of divalent cations (Rasmusson et al., 1999). A loose membrane attachment of hybrid NDB1 may be accounted for by interaction of this protein with divalent cations. To investigate the membrane association of NDB1 in plant mitochondria, antiserum was raised against the extreme C terminus of the NDB1 protein (Paper IV).

#### 4.4 Immunodetection of the external rotenone-insensitive NAD(P)H dehydrogenase

NDB antibodies raised against a polypeptide deduced from potato *ndb1*, a homologue to yeast and bacterial rotenone-insensitive NADH dehydrogenases, recognised NDB1 hybrid protein in *E.coli*. (Paper IV). NDB antiserum also immunoreacted specifically with a 61 kDa polypeptide in potato tuber mitochondria (Paper IV). Different molecular masses (180, 500, 600 and 700 kDa) of the native form of the protein suggest associations of varying numbers of NDB1 polypeptide subunits. Perhaps, *in vivo*, the enzyme operates as a multimeric oligomer, where different oligomeric forms influence the activity of the enzyme. This type of regulation is seen in NAD<sup>+</sup>-ME. NAD<sup>+</sup>-ME can exist as a heterodimer, heterotetramer and heterooctomer, each oligomeric form with distinct kinetic properties (Grover and Wedding, 1982).

The NDB antibody did not cross-react with proteins in peroxisomes and a microsomal fraction (Paper IV). A faint shadow at 50 kDa in chloroplasts correlates to the position of the large rubisco subunit, highly abundant in this organelle. NDB antiserum detected a single 61 kDa polypeptide in mitochondria isolated from various plant species (Paper IV).

Following sonication of mitochondria, immunodetected NDB1 protein was found only in soluble fractions (Paper IV). The release of putative external NAD(P)H dehydrogenases by sonication or osmotic swelling has previously been demonstrated (Luethy et al., 1995, Menz and Day, 1996a). By comparison, following digitonin solubilisation of the outer membrane of intact mitochondria, NDB1 was exclusively found in membrane fractions. Immunodetected NDB1 was associated with mitoplasts and to a lesser extent to the outer membrane fraction (Paper IV). Interestingly, the presence of NDB1 in the outer membrane fraction suggests that it had access to the outer membrane prior to disruption by digitonin. These results are consistent with the assignment of this protein as a peripherally attached, cytoplasmic facing enzyme of the inner mitochondrial membrane. As previously mentioned, NDB1 *in vivo*, may be able to form different oligomeric

configurations and move within the intermembrane space. Therefore, membrane associations in addition to oligomeric forms of the protein, may be a means of regulation for NDB1. However, whether these could be means of metabolic control remains to be investigated.

In order to correlate protein amounts with enzyme activities, beetroot slices were aged in the presence of calcium sulphate, previously shown to increase external NAD(P)H oxidation (Rayner and Wiskich, 1983, Cook-Johnson et al., 1999). NDin(NADH) is also induced by this treatment but to a lesser degree (Cook-Johnson et al., 1999, Paper IV). External NADH oxidation was more than 3-fold increased in mitochondria isolated from induced tissue (Paper IV). The rate of rotenone-insensitive malate oxidation was increased less than 2-fold. In contrast, oxidation of external NADPH hardly increased, as previously reported (Arron and Edwards, 1979). Antisera detected both NDA1 and NDB1 at levels of protein that in both cases are less than 2-fold increased in mitochondria isolated from induced tissue (Paper IV). The lack of correlation between the amount of NDB1 protein and external NADH oxidation activity in mitochondria isolated from induced tissue, indicate that NDB antiserum may instead recognise an external NADPH dehydrogenase in beetroot mitochondria. In order to determine whether NDB1 is a rotenone-insensitive NADPH dehydrogenase, *S. tuberosum ndb1* has been over expressed in *Nicotiana sylvestris*. This experiment is in progress.

Immunodetection of single polypeptides in mitochondria from various plant sources demonstrates a highly specific reaction, in which NDA and NDB antisera can be used with confidence for detection of the respective proteins in isolated mitochondria.

## **5 Physiological significance of alternative NAD(P)H:ubiquinone oxidoreductases in mitochondria**

### **5.1 Physiological role of internal NAD(P)H dehydrogenases**

Alternative internal NADH dehydrogenases oxidise NADH synthesised by the citric acid cycle, or other matrix NADH dehydrogenases, and can functionally substitute for complex I as demonstrated in mammalian cells (Seo et al., 1998). The *nd1* gene of *S. cerevisiae* was stably transformed into complex I-deficient mammalian cells, restoring ability of the respiratory chain to oxidise intramitochondrial NADH (Seo et al., 1998).

Plant mitochondria with complex I deficiencies show increased participation of the alternative NAD(P)H dehydrogenases (Gutierrez et al., 1997, Sabar et al., 2000). Complex I is a multimeric enzyme composed of ~ 30 subunits (Leterme et al., 1993, Herz et al., 1994, Rasmusson et al., 1994). Only nine subunits are synthesised in the mitochondria, whereas the remaining polypeptides, nuclear encoded, are synthesised in the cytoplasm and imported into the mitochondria (Rasmusson et al., 1999). Cytoplasmic male sterile (CMS) mutants in *N. sylvestris*, have a dysfunctional complex I, due to a mitochondrial DNA deletion in a complex I subunit gene (Gutierrez et al., 1997). Oxidation of intramitochondrial pyridine nucleotides was primarily rotenone-insensitive, indicating the survival of CMS plants was dependent on the participation of the rotenone-insensitive NAD(P)H dehydrogenases (Sabar et al., 2000). Increased activity of the internal rotenone-insensitive NADH dehydrogenase was seen in mitochondria isolated from rotenone-treated tobacco cell suspension cultures (Zhang et al., 2001), and beetroot slices aged in the presence of chloramphenicol, a mitochondrial protein synthesis inhibitor (Cook-Johnson et al., 1999).

In mitochondria which possess fully functional complex I activity, additional NAD(P)H dehydrogenases may participate in oxidation of excess respiratory substrate. For example, during photorespiration, glycine produced in large quantities is decarboxylated and deaminated by glycine decarboxylase in

mitochondria, with the concomitant reduction of  $\text{NAD}^+$  (Cohen-Addad et al., 1997). Up to 50% may be shuttled to the peroxisome, and the remainder oxidised by the internal NADH dehydrogenases in the respiratory chain (Krömer et al., 1992, Raghavendra et al., 1998).

NDA1 may participate in oxidation of NADH generated by glycine decarboxylase during photorespiration. Interestingly, expression of *nda1* of *S. tuberosum* is light regulated (Svensson and Rasmusson, 2001). The transcript level is highest during the early light period and lowest in the late afternoon (Svensson and Rasmusson, 2001). This photoperiodicity of an internal NADH dehydrogenase suggests a role in photorespiratory metabolism. Similar to *nda1*, mitochondrial photorespiratory enzymes, glycine decarboxylase and serine hydroxymethyltransferase are light regulated and show daily rhythms in expression (McClung et al., 2000). During photorespiration, reducing equivalents from glycine oxidation are available for rotenone-insensitive oxidation (Dry and Wiskich, 1985), and under these conditions NDA1 may function as an energy overflow bypass.

The activity of the alternative NAD(P)H dehydrogenases alters the redox balance in the mitochondrial matrix, and regulation of non-energy conserving pathways may have consequences for cellular metabolism (Palmer and Møller, 1982).

## **5.2 Physiological role of external NAD(P)H dehydrogenases**

Cytosolic  $\text{NAD(P)}^+$  reduced by glycolysis and/or the pentose phosphate pathway, is oxidised by external alternative NAD(P)H dehydrogenases (Douce and Neuburger, 1989). Mammalian mitochondria do not possess external NAD(P)H dehydrogenases. Instead, reducing equivalents from cytosolic NAD(P)H are transferred to matrix NAD(P)H by means of metabolite shuttles (Stryer, 1988). In *S. cerevisiae*, the absence of a malate-aspartate shuttle, most commonly found in mammalian mitochondria, correlates with the presence of an external NADH dehydrogenase (de Vries and Marres, 1987) However, enzyme activities for two

other shuttles have been demonstrated in yeast (Von Jagow et al., 1970, Rønnow et al., 1993).

In plant mitochondria, metabolite shuttles can be demonstrated *in vitro*. Operation of these shuttles may be important energetically, as oxidation of intramitochondrial NADH is associated with a higher production of matrix ATP than via external NADH dehydrogenases (Siedow and Day, 2000).

When amounts of ADP are low (state 4 conditions), alternative pathways (external and internal rotenone-insensitive NAD(P)H dehydrogenases including alternative oxidase) may maintain higher respiratory rates than the cytochrome pathway (Siedow and Day, 2000). Under these conditions, intermediates from the turnover of glycolysis and the citric acid cycle can be used for various biosynthetic pathways.

The physiological significance of the alternative enzymes in plant mitochondria which represent non-energy conserving wasteful pathways, still remains speculative. Further work is required to elucidate this.



## Populärvetenskaplig sammanfattning (Swedish summary)

Mitokondrier är små stavformade eller runda organeller (0.5-2  $\mu\text{m}$ ). De producerar huvuddelen av den energi som krävs för cellens livsuppehållande processer. Antalet mitokondrier per cell kan variera med vävnadens energibehov. Ett exempel är människans spermie som innehåller ett stort antal mitokondrier vilka behövs för producera den energi som åtgår när spermien simmar till ägget för befruktning. I en växts livscykel är pollenutvecklingen ett av de mest energikrävande stadierna där mitokondriella funktionsstörningar kan orsaka sterilitet.

De mitokondriella proteinerna kan delas in i två huvudgrupper: de som omvandlar energin i socker och fetter till för cellen användbara energirika molekyler, och de som avger energin i form av värme. Mitokondrierna, som ofta kallas cellens kraftverk, kan därför också sägas vara energislösande. De energislösande proteinernas fysiologiska roll är ännu inte fastlagd, men det är möjligt att de är inblandade i förbränning av ett överskott av kolhydrater.

Nedbrytningen av kolhydrater till koldioxid, vatten och energi i en cell kallas respiration. Vår arbete har gått ut på att undersöka i vilken omfattning energislösande proteiner deltar i respirationen. Dessa proteiner, så kallade NAD(P)H-dehydrogenaser, tar emot elektroner från NADH och NADPH som ursprungligen härrör från nedbrytning av kolföreningar producerade i mitokondrierna eller cytoplasman. I vår undersökning har vi kunnat påvisa att ett NADPH-specifikt, energislösande dehydrogenas deltar i respirationen (Artikel II).

När NADH och NADPH avger elektroner till NAD(P)H-dehydrogenaser bildas  $\text{NAD}^+$  och  $\text{NADP}^+$ . Dessa föreningar kan i sin tur bli nedbrutna och vi har identifierat mitokondriella proteiner som katalyserar denna reaktion ( $\text{NAD}^+$ -pyrophosphatase) (Artikel I).

I vår undersökning användes också specifika antikroppar för att detektera de energislösande proteinerna (Artikel IV).

## **Acknowledgements**

*my sincere thanks to:*

Professor Ian Max Møller who whilst visiting the Adelaide science laboratories inspired, encouraged and invited me to discover varied visions of Swedish culture, a new language, and to learn new and innovative ways in science.

Associate Professor Allan Rasmusson for his support, his inspiring discussions, and his sense of humour which I shall remember for many years.

Christina Nilsson who helped me peel all those kilos of potatoes and gave me invaluable help with the experiments.

Lena Carlsson who always watered the tobacco plants.

Lena Lundh for excellent technical assistance.

Per Westergren and Agne Gustavsson who always knew how to get things done.

Federico Gómez Galindo for his understanding.

Agnieszka, Sandra, Jose, Chris and Janet for their friendship.

Fredrik Johansson who helped with the translation of the Swedish summary.

Fernando Ortega Ojeda who helped during all those last minute computer crises.

Colleagues at the Departments of Plant Physiology and Plant Biochemistry for making my stay here so enjoyable.

My sister Erika for her inspiration, love and support

My parents' frequent visits to Sweden. Their support and inspiration never ceased.

*Lund, September 2001*

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