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PO Box 117
221 00 Lund
+46 46-222 00 00

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NAD(P) turnover in plant mitochondria

Stephanie C. Agius, Allan G. Rasmusson and Ian M. Møller[A]

Department of Plant Physiology, Lund University, Box 117, S-221 00 Lund, Sweden.
[A]Present address: Plant Biology and Biogeochemistry Department, Building 301, Risø National Laboratory, PO Box 49, DK-4000, Roskilde, Denmark. Corresponding author; email: ian.max.moller@risoe.dk

Abstract. An analytical procedure based on alkaline extraction and HPLC analysis was adapted for quantification of pyridine nucleotides in plant mitochondria. The amounts of NAD and NADP extracted from seven different species varied from 1.0 to 3.7 and 0 to 0.5 nmol (mg protein)$^{-1}$, respectively. Although NADP was found in four species, its reduced form was in all cases below the detection limit of 0.1 nmol (mg protein)$^{-1}$. The NAD pool was mainly oxidized in the absence of substrates. However, oxidation of substrates followed by anaerobiosis caused 50–92% NAD pool reduction, indicating that the majority of the NAD^+ was metabolically active. The NAD reduction level in potato tuber mitochondria oxidizing malate varied with assay conditions. The highest level of reduction (>80%) was reached at anaerobiosis, at pH 6.5 and 7.2, conditions favouring malic enzyme (ME), whereas the lowest reduction level (0%) was observed at pH 7.5, conditions favouring malate dehydrogenase (MDH). Mitochondria incubated at 0°C without respiratory substrate showed a loss of endogenous NAD^+ which correlated with a decline in the rate of oxidation of NAD^+-linked substrates. The lost NAD^+ was mainly recovered as breakdown products in both the surrounding medium and the mitochondria. When submitochondrial fractions were incubated with NAD^+ or $NADP^+$, the highest rate of $NAD(P)^+$ metabolism was detected in the outer membrane fraction. The metabolites detected, adenosine monophosphate (AMP), nicotinamide mononucleotide (NMN) and adenosine, imply that several enzymes involved in pyridine nucleotide degradation, including an NAD pyrophosphatase, are localized to the outer membrane.

Keywords: malate oxidation, mitochondria, NAD^+ metabolism, NAD^+ pyrophosphatase, NAD(P) amount, NAD(P) reduction level, phosphodiesterase.

Introduction

Discrete pools of pyridine nucleotides exist within subcellular compartments and specific carriers and shuttle systems are available for exchange of nucleotides across membranes (Laloi 1999). In mitochondria, NAD (NAD^+ + NADH) content may be regulated by NAD^+ uptake and loss but also by synthesis and breakdown. Dehydrogenases with different affinities for NAD^+ and NADH connect the Krebs cycle and the respiratory chain. The amount and reduction level of NAD in the mitochondrial matrix is therefore of central importance for the regulation of respiration (as well as many other processes) and its ability to fulfil a variety of roles in the plant such as the production of ATP and metabolic intermediates.

The content of NAD in the matrix of plant mitochondria has only been measured in a couple of species and tissues, and found to be in the range 1–6 nmol (mg protein)$^{-1}$ for mitochondria from potato tuber and mung bean hypocotyls (Tobin *et al.* 1980; Neuburger *et al.* 1985). This is equivalent to a concentration of 1–6 mM NAD, assuming a matrix volume of 1 μL per mg mitochondrial protein. Plant mitochondria can be loaded with NAD^+ (up to 5–10 nmol (mg protein)$^{-1}$) by incubation at room temperature with NAD^+, in the presence of a respiratory substrate to energize the inner membrane (Neuburger and Douce 1983). Exogenously added NAD^+ is transported across the inner membrane via a specific NAD^+ carrier and accumulates in the matrix space (Tobin *et al.* 1980). By comparison, mitochondria incubated in a medium lacking NAD^+ and respiratory substrate progressively lose NAD^+ in a temperature-dependent manner, suggested to be due to a reversal of the uptake transporter (Neuburger *et al.* 1985).

In plant tissues, NAD^+ can be enzymatically degraded and resynthesized in the pyridine nucleotide cycle (Wagner *et al.* 1986); however, none of the enzymes involved have been localized to the mitochondria. Thus, it is still not

Abbreviations used: ADP, adenosine diphosphate; AK, adenylated kinase; AMP, adenosine monophosphate; CCO, cytochrome-*c* oxidase; CCR, cytochrome-*c* reductase; EDTA, ethylenediamine tetraacetic acid; IMS, intermembrane space; K_m, equilibrium constant; MDH, malate dehydrogenase; ME, malic enzyme; MOPS, 4-morpholinopropanesulfonic acid; NMN, nicotinamide mononucleotide; OAA, oxaloacetate; OM, outer membrane; Rf, retention factor; SDS–PAGE, sodium-dodecyl sulfate polyacrylamide get electrophoresis; SMP, submitochondrial particle.

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known whether NAD breakdown and/or synthesis occurs in plant mitochondria, although Pearson and Wilson (1997) presented preliminary findings indicating that NAD^+ is metabolised by lysed potato tuber mitochondria. The breakdown products may be utilized for RNA or DNA synthesis as is known from the chloroplast (Pearson *et al.* 1993; Wilson *et al.* 1996).

Not only the amount of matrix NAD, but also its reduction level is an important regulatory parameter. It has long been known that oxidation of the NAD-linked substrate malate in plant mitochondria is very complex (Lance *et al.* 1967). Enzymatic product analyses showed that malate oxidation is carried out by two matrix enzymes. Malate dehydrogenase has an equilibrium constant (K_m) favouring formation of malate and NAD^+ and cannot maintain high NADH concentrations, unless oxaloacetate (OAA) is efficiently removed. Malic enzyme, by comparison, has K_m favouring formation of NADH, pyruvate and CO_2 and can therefore maintain the NAD pool more reduced (Palmer *et al.* 1982; Neuburger *et al.* 1984). The relative activities of these two enzymes and the presence of two NADH dehydrogenases in the inner membrane facing the matrix — the rotenone-sensitive, proton-pumping complex I with a low K_m(NADH) and the rotenone-insensitive, non-proton-pumping NADH dehydrogenase with a high K_m(NADH) — were shown to cause these complex patterns of malate oxidation (Møller and Palmer 1982; Palmer *et al.* 1982; Neuburger *et al.* 1984; Møller and Lin 1986). Initially, the reduction level of NAD was estimated indirectly from analyses of other metabolites (Tobin *et al.* 1980; Palmer *et al.* 1982), but later, semiquantitative fluorescence measurements, assumed to reflect the amount of NADH in the matrix, did indeed vary as expected (Neuburger *et al.* 1985). However, direct measurements of the amounts of NAD^+ and NADH were never carried out.

Early enzymatic measurements of nucleotides showed that not only NAD but also NADP was present in unpurified potato mitochondria at a level six times lower than NAD (Brinkman *et al.* 1972). In contrast, Roberts *et al.* (1997) reported that the amount of NADP was twice that of NAD due mainly to a low NAD content. It has been suggested that NADP has a multitude of potential roles in plant mitochondria (Møller and Rasmusson 1998). One such role is contributing to electron transport through the activity of an NADPH dehydrogenase facing the matrix (Rasmusson and Møller 1991; Melo *et al.* 1996), which is able to contribute to the oxidation of malate (Agius *et al.* 1998). In view of this, it was relevant to investigate whether fluorescence previously assumed to be caused solely by NADH (Neuburger *et al.* 1985) could partly be due to NADPH, which has fluorescence characteristics similar to NADH (Tobin *et al.* 1980; Neuburger *et al.* 1984). Also the large discrepancy in the literature concerning the relative amounts of NAD and NADP called for an independent investigation.

In the present study, we have adapted an HPLC method to identify and quantify in plant mitochondria the nucleotides NAD^+, NADH, $NADP^+$ and NADPH and their various potential breakdown products. We have measured the content and reduction levels of NAD and NADP in a wide range of plant mitochondria incubated under different respiratory states. We have further reinvestigated the loss of NAD^+ from potato tuber mitochondria and found that NAD^+ lost from mitochondria on cold storage is also degraded, and that nicotinamide mononucleotide, AMP and adenosine can be detected as the main breakdown products. Finally, we localized the enzymes responsible for $NAD(P)^+$ breakdown to the outer mitochondrial membrane (OM).

Materials and methods

Isolation of mitochondria

Mitochondria were purified as reported for the following species and tissues: potato (*Solanum tuberosum* L. cv. Bintje) tubers according to Struglics *et al.* (1993); potato (*Solanum tuberosum* L. cv. Grata) tubers incubated with apples for 48 h to induce alternative oxidase activity according to Lidén and Åkerlund (1993); Jerusalem artichoke (*Helianthus tuberosus* L.) tubers according to Lidén and Møller (1988); red beetroots (*Beta vulgaris* L. cv. Nina) according to Rasmusson *et al.* (1994); *Arum italicum* flower spadices collected in the Royal Botanic Gardens, Adelaide, Australia in the late ε-stage according to Day *et al.* (1985); 7-d-old etiolated maize (*Zea mays* L.) seedlings according to Moore and Proudlove (1983); leaves from 10–14-d-old pea (*Pisum sativum* L. cv. Oregon) seedlings according to Day *et al.* (1985). Crude mitochondria (Van der Bergen *et al.* (1991) from sweet potato (*Ipomoea batatas* L.) roots were purified as described in Struglics *et al.* (1993).

Measurement of oxygen consumption

Respiration was measured polarographically as oxygen consumption using a Rank Brothers oxygen electrode (Rank Brothers, Cambridge, UK) in reaction medium, containing 0.3 M sucrose, 20 mM 4-morpholinopropanesulfonic acid (MOPS) (pH 7.2), 5 mM KH_2PO_4, 2.5 mM $MgCl_2$, and 0.01% (w/v) bovine serum albumin. To facilitate measurements at very high protein concentrations (5–8 mg mL^{-1}), assays were conducted at 15°C, unless otherwise stated.

For continuous state 3 conditions, 10 units hexokinase, 0.05 mM adenosine diphosphate (ADP), 30 mM glucose were added to mitochondria, whereas for state 4 and anaerobiosis, 0.05 mM ADP was added to give an initial state 3–state 4 cycle. Antimycin A (5 μM), 1 mM KCN and 100 μM n-propylgallate were added to anaerobic incubations to minimize reoxidation of NAD(P)H through the respiratory chain prior to extraction. Mitochondrial respiratory states are defined according to Chance and Williams (1956).

Long-term incubation of mitochondria

Mitochondria (70–100 mg mL^{-1}) were stored on ice in an isotonic medium (0.3 M mannitol, 10 mM MOPS, pH 7.2) for a period of 48 h under constant agitation to prevent anaerobiosis.

For analysis of nucleotides and metabolites in the mitochondria and surrounding medium, 5 mM $MgCl_2$ was added, and the mitochondria pelleted at 39 200 *g* for 10 min The supernatant was frozen in liquid nitrogen and stored at –80°C until analysis. The pellet was resuspended in wash medium (0.3 M mannitol, 10 mM MOPS (pH 7.2), 1 mM ethylenediamine tetraacetic acid (EDTA) and 5 mM $MgCl_2$) and recentrifuged. Mitochondria were resuspended in wash medium minus $MgCl_2$ and extracted.

Nucleotide and metabolite extraction and determination by HPLC

Mitochondrial subfractions were extracted essentially as described by Stocchi *et al.* (1985) with the following modifications. Samples (2 mg protein) were mixed with ice-cold 0.5 M KOH and vortexed for 1 min. Ice-cold H_2O was added to give a final KOH concentration of 0.125 M and the solubilized suspension filtered through a CF 50-kDa Amicon membrane by centrifugation at 7500 *g* for 10 min The pH of the filtered solution was adjusted to 7.2 by addition of 1 M KH_2PO_4. Samples were frozen in liquid nitrogen and stored at –80°C until analysis. Immediately before HPLC analysis, samples were adjusted to pH 6.5 and passed through 0.22-µm filters. Extraction of nucleotides was carried out from 2 mg of mitochondrial protein, since higher protein loads frequently resulted in blocking of the filters used.

Samples were analysed by reverse-phase HPLC on a 5-µm Supelcosil LC-18 column (25 cm × 4.6 mm i.d., Supelco, Belfonte, PA, USA) using a gradient buffer and flow rate of 1.3 mL min^{-1}. The gradient consisting of buffer A (0.2 M KH_2PO_4, pH 6.0) and buffer B (0.2 M KH_2PO_4 containing 10% (v/v) CH_3OH, pH 6.0) was run for 31.5 min as follows: 100% A for 9 min, 0–25% B for 6 min, 25–90% B for 2 min 30 s, 90–100% B for 2 min, 100% B for 6 min, 100% A for 6 min. Separation was conducted at 30°C and detection monitored at 259 nm. Standards were always run on the same day to ensure an accuracy of within 2% for retention factor (Rf) values.

Isolation of submitochondrial fractions

Subfractionation of mitochondria was carried out essentially as described by Ragan *et al.* (1987). Mitochondria were diluted to a concentration of 1.1 mg digitonin per 10 mg protein in 20 mM Tris–HCl (pH 7.4), 350 mM mannitol and 6 mM EDTA. After 20 min of constant agitation on ice, two volumes of buffer containing 20 mM Tris–HCl, pH 7.4, 300 mM mannitol were added and the mitoplasts pelleted at 27000 *g* for 15 min The supernatant was spun at 300000 *g* for 60 min to obtain the OM (pellet) and intermembrane space (IMS) fraction (supernatant). Submitochondrial particles (SMP) and the matrix fraction were isolated from potato tuber mitochondria as described by Rasmusson and Møller (1991). Matrix and IMS fractions were concentrated using 10 kDa cut-off filters.

Enzyme assays and protein determination

Cytochrome-*c* oxidase (CCO, EC 1.9.3.1) and NAD^+-malate dehydrogenase (EC 1.1.1.37) activities were assayed according to Møller *at al.* (1987). Activities were determined in the presence or absence of 0.025% (w/v) Triton X-100 (TX). The percentage latency was calculated as 100 × [(rate + TX) – (rate – TX) / (rate + TX)] (Rasmusson and Møller 1991).

Adenylate kinase (AK, EC 2.7.4.3) and antimycin A-insensitive NADH-cytochrome-*c* reductase (CCR, EC 1.6.99.3) activities were measured essentially as described by Day and Wiskich (1975) and Day *et al.* (1979), respectively.

Mitochondrial protein was measured according to Lowry *et al.* (1951), with bovine serum albumin as a standard.

Immunoblot for detection of porin

Proteins were separated by 12% (w/v) sodium-dodecyl sulfate polyacrylamide get electrophoresis (SDS–PAGE) (Laemmli 1970), electrotransferred to a PVDF membrane and immunoblotted with monoclonal antibodies directed against *Dictyostelium discoideum* mitochondrial porin. Antibody interaction was detected using the ECL-western-blotting analysis system (Amersham Pharmacia Biotech, Uppsala, Sweden).

Results and discussion

An HPLC method was adapted for the identification and quantification of pyridine and adenine nucleotides

The determination of NAD(P) usually entails separate acidic and alkaline extractions since oxidized and reduced pyridine nucleotides have different stabilities (Lowry *et al.* 1961). This process is laborious and has the disadvantage that separate extractions are needed to measure amounts of $NAD(P)^+$ and NAD(P)H, thus increasing experimental error in the determination of the reduction level. In the present paper, we have adapted an analytical method with alkaline extraction, followed by HPLC analysis, facilitating the simultaneous detection of oxidized and reduced pyridine nucleotides as well as adenine nucleotides in isolated plant mitochondria (Stocchi *et al.* 1985).

HPLC chromatograms of a standard mixture of pyridine and adenine nucleotides show excellent separation of all compounds (Fig. 1). The recoveries were for pyridine nucleotides $NADP^+$, NADPH, NAD^+ and NADH: 91, 91, 82 and 93%, respectively, (the percentage recoveries analysed as internal controls for pyridine nucleotides were similar); for adenine nucleotides ATP, ADP and AMP: 95, 93 and 100%, respectively; for adenine nucleosides ADP-ribose and adenosine: 99 and 96%, respectively; and NMN, 86%. Identification of peaks by Rf values and quantification from peak area was concluded to be reliable.

The amounts of mitochondrial NAD and NADP differ between plant species and tissues

Mitochondria isolated from a range of species and tissues were incubated in the absence of substrates (state 1) to provide relatively oxidized conditions or in the presence of a cocktail of $NAD(P)^+$-linked TCA-cycle substrates followed by anaerobiosis (state 5) to maximize reduction. The total NAD pool was similar during respiratory state 1 and 5,

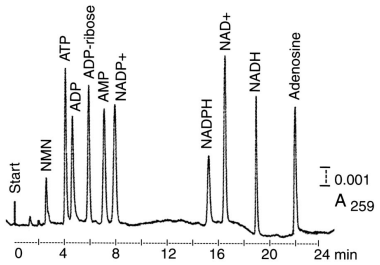

Fig. 1. HPLC analysis of pyridine nucleotides and ADP-ribonucleotides using reverse-phase HPLC. Standards (100 pmol of each): NMN, ATP, ADP, ADP-ribose, AMP, $NAD(P)^+$, NAD(P)H and adenosine were separated by reverse-phase chromatography as described in 'Materials and methods'.

indicating that the extraction method was reliable (Table 1). The total NAD pool varied from 0.93 nmol mg^{-1} in induced potato tuber mitochondria to 3.7 nmol mg^{-1} in pea leaf mitochondria, equivalent to a matrix concentration of 0.9–3.7 mM NAD, assuming a matrix volume of 1 μL per mg mitochondrial protein. The results obtained are consistent with previous studies where NAD^+ and NADH were extracted separately and quantified by enzymatic analyses (Brinkman *et al.* 1972; Tobin *et al.* 1980; Neuburger and Douce 1983). The NAD content in potato tuber mitochondria isolated from uninduced tubers was almost three times higher than in mitochondria isolated from induced tubers (Table 1). This difference may be due to the cultivar used or reflect different metabolic requirements between the two tissues for this essential cofactor.

In state 1, intramitochondrial NAD was mainly found in the oxidized form. In contrast, substrate oxidation followed by anaerobiosis in most cases shifted the redox state to a much higher reduction level (Table 1) demonstrating that the majority of the NAD was metabolically active. Similarly, glycine oxidation by pea leaf mitochondria under state 5 conditions caused a substantial conversion of matrix NAD^+ into NADH (Wigge *et al.* 1993).

$NADP^+$ was detected in mitochondria from *S. tuberosum* L. cv. Bintje, *I. batatas* L., *H. tuberosus* L., *Z. mays* L. and *P. sativum* L. at 0.2–0.5 nmol mg^{-1} protein corresponding to 10–30% of the size of the total NAD pool (Table 1). However, in contrast to what was observed for NAD, the state 5 conditions were ineffective in reducing the NADP pool to levels detectable by HPLC analysis (Table 1). This was surprising considering previous results implicating NADPH involvement in malate oxidation (Agius *et al.* 1998*a*), but even NADPH concentrations below our detection limit of 0.1 nmol mg^{-1} (corresponding to a concentration of 100 μM) would be sufficient to give substantial activity of inner membrane matrix-facing rotenone-insensitive NAD(P)H dehydrogenase (ND_{in}), since its K_m (NADPH) is only 25 μM (Rasmusson and Møller 1991). In comparison, complex I has a high K_m of about 1 mM for NADPH and should not be active in NADPH oxidation. Earlier investigations have found 0.2–0.7 nmol mg^{-1} NADP in mitochondria from pea leaves and potato tubers (Brinkman *et al.* 1972; Wigge *et al.* 1993; Roberts *et al.* 1997). The low amounts of NADP found in the present study are consistent with the results of Brinkman *et al.* (1972) for washed potato tuber mitochondria. In contrast, Roberts *et al.* (1997) found twice the amount of NADP as compared with NAD by quantifying $NAD(P)^+$ directly and NAD(P)H by its degradation products detected in acidic extracts of purified potato tuber mitochondria by ^{31}P-nuclear magnetic resonance (NMR). The disparity in amounts of cofactors may be due to differences in mitochondrial purity, assay conditions prior to the extraction of nucleotides, analytical methods used, and/or to seasonal variation in potato tubers (Brinkman *et al.* 1972; Neuburger and Douce 1983). The low amounts of mitochondrial NADP found in this investigation prevent accurate quantification of this nucleotide by HPLC.

Table 1. NAD(P) content in mitochondria from various species and tissues measured in respiratory states 1 and 5
Mitochondria isolated from various tissues were incubated in reaction medium, pH 7.2 (i.e. state 1) or in reaction medium plus 10 mM malate, 10 mM glutamate, 10 mM isocitrate, 100 μM adenosine diphosphate, 0.5 mM coenzyme A and 5 mM dithiothreitol. After 5 min anaerobiosis (i.e. state 5), Antimycin A (5 μM), 1 mM KCN and 100 μM n-propyl gallate were added. Samples were extracted and analysed as described in 'Materials and methods'. All replicates are from one preparation of mitochondria; however, similar trends were seen with 2–5 independent preparations except for *Ipomoea batatas* and *Arum italicum* where only one preparation was tested. Values are expressed as mean ± s.d. in pmol (mg protein)$^{-1}$. NADPH was always below the detection limit of 100 pmol (mg protein)$^{-1}$

Source of mitochondria	Respiratory state	Replicates	Total NAD	% NAD reduced	$NADP^+$
Solanum tuberosum cv. Bintje tubers	state 1	4	2530 ± 70	<4	515 ± 59
	state 5	2	3020 ± 222	81 ± 1	<100
S. tuberosum cv. Grata induced tubers	state 1	3	930 ± 111	<11	<100
	state 5	2	1080 ± 38	52 ± 1	<100
Ipomoea batatas tubers	state 1	4	1790 ± 265	<6	230 ± 33
	state 5	6	2700 ± 460	71 ± 2	230 ± 33
Zea mays coleoptiles	state 1	3	1650 ± 177	<6	470 ± 36
	state 5	3	1600 ± 62	80 ± 3	<100
Arum italicum spadices	state 1	5	2820 ± 391	50 ± 4	<100
	state 5	5	2960 ± 710	58 ± 3	<100
Pisum sativum leaves	state 1	4	3740 ± 295	<3	420 ± 12
	state 5	4	2540 ± 80	75 ± 1	380 ± 51
Beta vulgaris roots	state 1	3	1870 ± 134	<6	240 ± 206
	state 5	2	2290 ± 199	92 ± 1	<100
Helianthus tuberosus tubers	state 1	3	1840 ± 51	31 ± 1	240 ± 204
	state 5	3	1610 ± 176	59 ± 1	530 ± 46

The reduction level of matrix NAD varies as a function of the in vitro *metabolic conditions*

Previous measurements of intramitochondrial pyridine nucleotides using fluorescence spectroscopy (Neuburger *et al.* 1984) have several potential weaknesses: (i) the method cannot discriminate between NADH and NADPH. Considering the relatively high amount of mitochondrial NADP reported by Roberts *et al.* (1997), it was therefore unclear to what extent the two reduced coenzymes contributed to produce this fluorescence; (ii) it is only semiquantitative, since bound and free NADH do not have the same fluorescence yield (Paul and Schneckenburger 1996); and (iii) in addition, an inactive pool of NAD^+ will be invisible. Since NADPH was below the detection limit of about 0.1 nmol (mg protein)$^{-1}$ (equivalent to 0.1 mM in the matrix) in all mitochondrial extracts (results not shown), we can conclude that Neuburger *et al.* (1984) were correct in assuming that the fluorescence mainly reflected NADH. The remaining discussion in this section will focus on the NAD reduction level.

Potato tuber mitochondria were exposed to assay conditions to reproduce the range of conditions used previously (Tobin *et al.* 1980; Palmer *et al.* 1982; Neuburger *et al.* 1984). The reduction level of NAD during malate oxidation was investigated under three different conditions: (a) at pH 6.5 where only ME is active, (b) at pH 7.2, where MDH is active, with glutamate present to remove the MDH product OAA and (c) at pH 7.5, where only MDH is active, without the addition of an OAA-removing system.

Generally the rate of malate oxidation at 15°C was higher (Fig. 2*A*) and the NAD reduction level (Fig. 2*B*) was lower in state 3, than in states 2 and 4, consistent with previous results obtained at higher temperatures (Neuburger *et al.* 1984). The very low oxidation rate at pH 7.5 compared with that at pH 6.5 and 7.2 may indicate that MDH is particularly sensitive to lower temperature (Fig. 2*A*).

In the presence of malate, reduction of the NAD pool could be observed at all pH tested and the highest levels of reduction were seen under anaerobiosis (state 5) (Fig. 2*B*). The redox state of the pyridine nucleotide pool varied, depending on assay conditions.

The lowest level of reduction of the NAD pool was always seen at pH 7.5, in all respiratory states (Fig. 2*B*). This was most apparent under state 3 conditions, at pH 7.5, where no NADH was detectable. The detection limit for NADH was about 70 pmol mg^{-1}, corresponding to a maximal reduction level of 5%. Also in states 2 and 4, the NAD pool was less reduced at higher pH (Fig. 2*B*). The difference in maximum reduction levels at the different pH used most likely reflects the equilibria of the ME and MDH reactions.

The addition of rotenone under state 3 conditions decreased the rate of malate oxidation by 26–45%. In parallel, the reduction state of NAD increased from 46 to 53% at pH 6.5 and from 18 to 32% at pH 7.2. No NADH

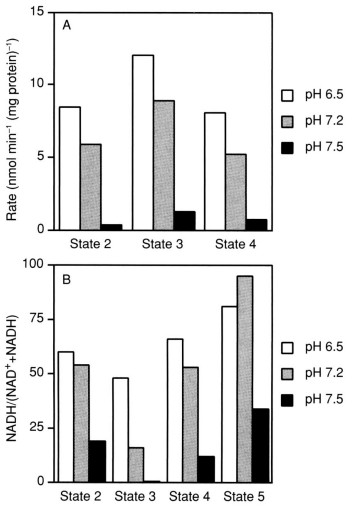

Fig. 2. Oxygen consumption (*A*) and reduction level of NAD (*B*) in potato tuber mitochondria oxidising malate. Malate (10 mM) oxidation was carried out at 15°C in the presence of 0.5 mM coenzyme A at pH 6.5 and 10 mM glutamate at pH 7.2. To all assays 1 mM ethylene glycol-bis(aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid was added. Samples were taken out from the oxygen electrode for extraction when 40% oxygen had been consumed, except for anaerobiosis (state 5) where the incubation was left for 10 min after O_2 depletion before sampling. Antimycin A at 5 μM was added to anaerobic incubations to minimise reoxidation of NAD(P)H through the respiratory chain prior to extraction. For analysis of the redox state of the NAD pool (B), samples were extracted and analysed as described in 'Materials and methods'. Values are means of two replicates on one mitochondrial preparation; however, similar results were obtained on another preparation.

was detected at pH 7.5 in the presence or absence of rotenone (results not shown). Thus, data obtained by direct quantification support fluorescence analyses, indicating an increased NAD^+ reduction on rotenone addition, to potato tuber mitochondria oxidizing malate (Tobin *et al.* 1980; Palmer *et al.* 1982; Neuburger *et al.* 1984). The low reduction levels at pH 7.5 should cause substrate restrictions for the high K_m rotenone-insensitive ND_{in}(NADH) (Møller and Palmer 1982; Rasmusson and Møller 1991), since a substantial part of the NADH should be enzyme bound and not free for catalysis (Wigge *et al.* 1993).

In general, results on the reduction level of NAD obtained by extraction and HPLC quantification confirm previous data obtained by fluorescence measurements, and are in agreement with interpretations based on the usually unstated assumption that free and bound matrix NAD behave similarly (Tobin *et al.* 1980; Møller and Palmer 1982; Palmer *et al.* 1982; Neuburger *et al.* 1984; Møller and Lin 1986).

The decline in NAD(P)-dependent respiratory rates during long-term incubation of potato tuber mitochondria is due to metabolism and transport of NAD$^+$

Plant mitochondria stored at 0°C for 48 h or at 25°C for 2 h in the absence of respiratory substrate, progressively lose the majority of their NAD$^+$ (Neuburger and Douce 1983; Neuburger *et al.* 1985). The fate of this lost NAD$^+$ has not been established although Neuburger and Douce (1983) mentioned that NAD$^+$ released could be recovered in the external medium if the pH was maintained below 7.0.

Freshly isolated potato tuber mitochondria stored at 0°C for 48 h remained highly intact as monitored by the latency of CCO and MDH. The respiratory activity of mitochondria oxidizing malate, 2-oxoglutarate (2-OG) and isocitrate under state 3 conditions declined over time, but the activity was fully restored by the addition of NAD$^+$. Succinate oxidation was unaffected. These results (not shown) are consistent with previous reports (Neuburger and Douce 1983; Neuburger *et al.* 1985).

The NAD$^+$ content of potato tuber mitochondria declined from 1.8 to 0.6 nmol mg^{-1} over 48 h at 0°C but lost NAD$^+$ was not recovered in the surrounding medium (Fig. 3*A*). The two NAD$^+$ metabolites, NMN and AMP were detected in significant amounts. In the mitochondria, the amount of AMP increased during the first 24 h and then decreased while NMN was close to the detection limit throughout (Fig. 3*B*). In the surrounding medium, NMN accumulated almost linearly with time while AMP and adenosine were barely detectable with a weak upward trend (Fig. 3*C*). The amounts of ADP and ATP in mitochondria and the surrounding medium were low at all time points tested. In addition to the NAD$^+$ metabolites present in mitochondria and surrounding medium, some unidentified peaks were found during the incubation (results not shown).

The rate of passive diffusion of NAD$^+$ has been reported to be dependent on temperature and the concentration of free NAD in the mitochondrial matrix (Neuburger *at al.* 1985). However, nothing is known about the fate of NADP$^+$. In order to investigate this, the potential loss of NADP$^+$ in potato tuber mitochondria was followed over time. In contrast to the notable loss of NAD$^+$ from the mitochondrial matrix incubated at 0°C for 48 h in the absence of respiratory substrate, no significant loss of NADP$^+$ was detected (results not shown).

In higher plants, three NAD-catabolising activities have been identified in partially purified enzyme extracts from tobacco roots (Wagner *et al.* 1986): NAD glycohydrolase (EC 3.2.2.5) cleaves between the nicotinamide group and the ribose producing nicotinamide and ADP-ribose, phosphodiesterase (EC 3.1.4.1) cleaves at the phosphate group yielding adenosine, nicotinamide ribose and pyrophosphate, whereas NAD pyrophosphatase (EC 3.6.1.22) cleaves the pyrophosphate bond yielding AMP and NMN

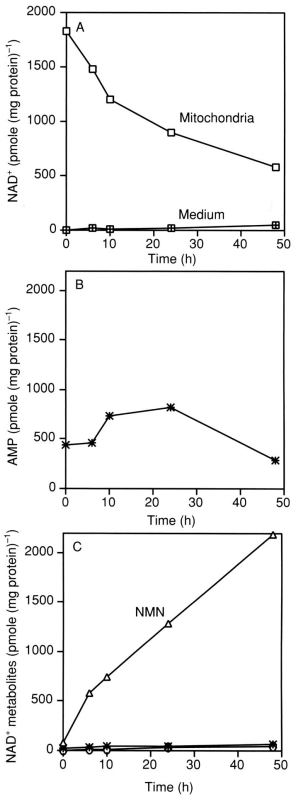

Fig. 3. Degradation of NAD$^+$ in potato tuber mitochondria stored on ice for 48 h. (*A*) NAD$^+$ content in mitochondria and surrounding medium (*B*) AMP in mitochondria and (*C*) metabolites in the surrounding medium. *, AMP; o, adenosine. NMN and adenosine were barely detected in the mitochondria with no clear trend.

(Fig. 4) (Wagner *et al.* 1986). The metabolite pattern shown in Fig. 3 indicates that NAD pyrophosphatase and to a lesser extent phosphodiesterase activities are present in potato tuber mitochondria (Figs 3 and 4). It was not possible to ascertain whether NAD glycohydrolase contributed to NAD$^+$ metabolism in the mitochondria due to the presence of some ADP-ribose in nucleotide extractions from control incubations (not shown), indicative of non-enzymatic degradation of NAD$^+$ to ADP-ribose.

The presence of NMN in the supernatant and AMP in the pellet raise the question of where NAD$^+$ is broken down? NAD$^+$ may leave the mitochondria and be metabolized by

enzymes in the OM followed by uptake of AMP or an AMP-containing metabolite. Alternatively, NAD^+ catabolism occurs in the matrix and NAD^+ metabolites, specifically NMN, passively diffuse out of the mitochondria into the surrounding medium. The results in Fig. 3 clearly show that NAD^+ is degraded. However, analyses of metabolites from both the mitochondria and surrounding medium were unable to clarify the localization of NAD^+-catabolising enzymes. Therefore, mitochondria were subfractionated, incubated with NAD^+ or $NADP^+$, and the NAD(P) $^+$-metabolites analysed.

The OM contains several enzymes involved in NAD(P)$^+$ breakdown

Freshly isolated and highly intact potato tuber mitochondria were either sonicated to obtain SMPs and soluble proteins (i.e. IMS plus matrix), or selectively solubilized with digitonin to give OM, IMS and mitoplasts (i.e. mitochondria partially stripped of OMs). To ascertain the relative purity of the fractions, specific and total activities of various markers were determined (Table 2). CCR and porin were highly enriched in the outer membrane fraction (5–10-fold increased specific activity) that contained little inner membrane, as measured by CCO activity, or soluble protein, as measured by MDH activity (Fig. 5, Table 2). AK activity was associated with membrane fractions, consistent with earlier reports (Day *et al.* 1979), making it an unsuitable marker for the IMS. The IMS was contaminated with matrix (49%) as seen from MDH activity but contained very little OM (porin) or inner membrane (CCO). The mitoplasts had lost some OM as seen from the less intense band in the porin blot compared with intact mitochondria, in addition to the decreased CCR specific activity and lower CCO latency, but had intact inner membranes as judged by the high MDH latency. SMPs were highly enriched in the inner membrane marker CCO and contained little matrix (MDH); however, some OM was also present as seen from the porin blot (Fig. 5). The soluble protein fraction contained virtually no membranes (low CCO activity and no porin detectable). The percentage of IMS in the soluble fraction could not be determined because of the absence of an IMS marker, but considering the very small amount of IMS protein in intact mitochondria, contamination should be negligible.

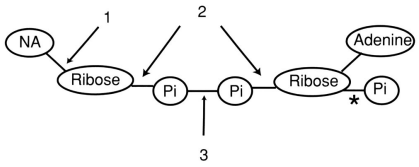

Fig. 4. Enzymatic cleavage of NAD^+. *1*, NAD glycohydrolase gives nicotinamide and ADP-ribose; *2*, phosphodiesterase gives adenosine, nicotinamide ribose and pyrophosphate and *3*, NAD pyrophosphatase gives AMP and NMN. The 2′-phosphate denoted by an asterisk is not present in NAD^+, only in $NADP^+$. Figure adapted from Hagen and Ziegler (1997).

The above fractions were incubated with NAD^+ or $NADP^+$, samples taken out at 0, 5 and 60 min, and NAD(P)$^+$, and their potential breakdown products extracted from the reaction medium, identified and quantified by HPLC analyses. Results from 0 and 60 min are presented in Tables 3 and 4, although data for 5 min are in general consistent with trends seen for 60 min. The HPLC elution profiles are shown for the OM fraction in Fig. 6. The rate of NAD^+ disappear-

Table 2. Marker enzyme activities of individual subfractions of potato tuber mitochondria

Mitochondria were selectively solubilized with digitonin to obtain outer membrane (OM), intermembrane space (IMS) and mitoplasts, or sonicated to give submitochondrial particles (SMP) and soluble protein fraction (IMS + matrix). Marker enzyme activities for OM (antimycin A-insensitive NADH-cytochrome-*c* reductase, CCR), IMS (adenylate kinase, AK), matrix (malate dehydrogenase, MDH) and inner mitochondrial membranes (cytochrome-*c* oxidase, CCO) were determined as described in 'Materials and methods'. Values are expressed as μmol min^{-1} mg^{-1} and represent mean ± s.d., n = 2–5. Similar results were obtained with one other mitochondrial preparation. n.d., not determined. Total relative recovery and latency are given as %

Enzyme assay	Parameter	Intact mitochondria	Digitonin fractionation OM	IMS	Mitoplasts	Fractionation by sonication SMP	Soluble protein
CCO	Total relative recovery	100	<1	<1	55	26	4
	Specific activity	5.94 ± 0.064	<0.0001	<0.0001	5.94 ± 0.15	11 ± 1.6	0.012
	Latency	99	n.d.	n.d.	79 ± 0.6	92 ± 4	n.d.
MDH	Total relative recovery	100	12	16	62	11	134
	Specific activity	7.12 ± 1.3	2.17 ± 0.22	16.7	8.1 ± 0.63	3.72	34.2 ± 0.2
	Latency	97 ± 1.1	n.d.	n.d.	96 ± 2.1	n.d.	n.d.
CCR	Total relative recovery	100	2	9	46	24	30
	Specific activity	0.040 ± 0.002	0.198	0.035 ± 0.0034	0.033 ± 0.002	0.056 ± 0.002	0.05 ± 0.003
AK	Total relative recovery	100	0.4	0.9	69	41	3
	Specific activity	0.439 ± 0.01	0.43	0.039 ± 0.002	0.551 ± 0.01	1.06 ± 0.05	0.0534 ± 0.01

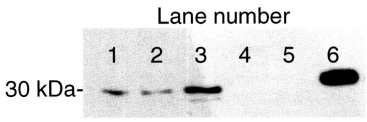

Fig. 5. The amount of the outer membrane protein, porin, in potato tuber mitochondria and submitochondrial fractions as determined by western blotting. Lane 1, mitochondria; lane 2, mitoplasts; lane 3, SMP; lane 4, IMS; lane 5, soluble protein (IMS + matrix); lane 6, OM. Total protein loaded per lane was 20 μg.

ance catalysed by OM was 1300 pmol min^{-1} (mg protein)$^{-1}$, seven times that of any other fraction (Table 3). Some NAD^+-catabolising activity was observed with mitoplasts and SMPs, both of which contained some OM (Table 3, Fig. 5). The rate of NAD^+ breakdown was lower in intact mitochondria than in mitoplasts possibly due to activation of NAD^+-degrading enzymes by digitonin solubilization. No NAD^+ catabolism was observed with the soluble protein fraction (results not shown).

In parallel with the disappearance of NAD^+, NMN, AMP and adenosine appeared (Fig. 6, Table 3). In the OM fraction, the amount of NMN was almost stoichiometric with NAD^+ and so was the sum of AMP and adenosine. Adenosine appeared more slowly than AMP (based on the 5-min data). This indicates that NAD^+ was initially split into NMN plus AMP by an NAD^+ pyrophosphatase and the resulting AMP converted into adenosine by a phosphatase (see Fig. 4). However, we cannot exclude the possibility that some of the adenosine was formed directly by a phosphodiesterase since we did not have nicotinamide ribose, the other breakdown product resulting from phosphodiesterase activity, amongst our standards. After 5- and 60-min incubation of OM with NAD^+, in addition to NMN, AMP and adenosine, three unidentified peaks appeared (Fig. 6), one of which may be nicotinamide ribose. A preliminary report using lysed potato tuber mitochondria also demonstrated rapid breakdown of NAD^+ and accumulation of AMP and adenosine, in addition to unidentified peaks (Pearson and Wilson 1997).

When the OM fraction was incubated with $NADP^+$, a similar pattern of metabolism was observed as for NAD^+;

Table 3. Metabolites detected after incubation of NAD^+ with submitochondrial fractions from potato tuber mitochondria

NAD^+ and degradation products produced by submitochondrial fractions incubated in reaction medium with 100 μM NAD^+ for 0 and 60 min at 30°C. Adenosine diphosphate (ADP) and ATP were detectable; however, no change in the amounts was seen. $NADP^+$, NADH and NADPH were not detected in any incubation. Samples were extracted and analysed as described in 'Materials and methods'. +, indicates a net increase; –, indicates a net decrease. Values represent averages of 2–5 replicates. Similar results were obtained with one other mitochondrial preparation. NMN, nicotinamide mononucleotide; AMP, adenosine monophosphate. The start point for mitoplast incubations at '0 min' was 5 min

Fraction	Metabolite	Amount (pmol mg^{-1}) 0 min	60 min	Rate (pmol mg^{-1} min^{-1})
Mitochondria	NMN	0	4200	+69
	AMP	440	2970	+42
	NAD^+	84 000	78 000	–108
	Adenosine	0	391	+7
Outer membrane (OM)	NMN	0	83 000	+1390
	AMP	0	26 800	+450
	NAD^+	79 000	4100	–1250
	Adenosine	0	45 000	+750
Intermembrane space	NMN	510	1410	+15
	AMP	970	2250	+21
	NAD^+	71 000	77 000	+95
Mitoplasts	NMN	660	7900	+131
	AMP	1140	8400	+131
	NAD^+	83 000	73 000	–182
	Adenosine	0	3360	+61
SMP	NMN	0	5240	+87
	AMP	410	9000	+143
	NAD^+	84 000	77 000	–121
	Adenosine	0	322	+5

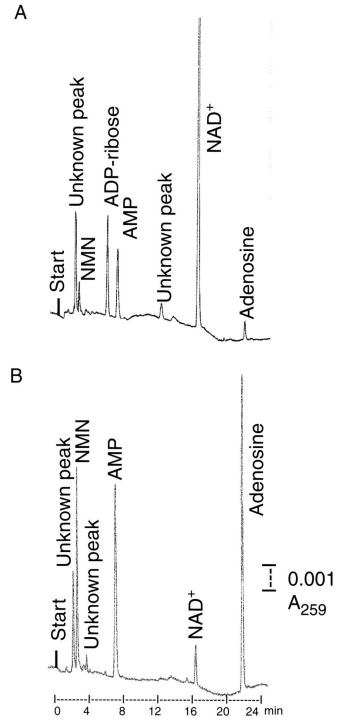

Fig. 6. HPLC chromatograms of NAD^+ incubated with outer membrane fraction. 99% pure NAD^+ (100 μM) was incubated with OM (0.1 mg) in reaction medium for 5 (*A*) or 60 min (*B*) at 30°C. Similar results were obtained from one other mitochondrial preparation.

however, enzymatic activities were 5–10 times lower (Table 4). Interestingly, adenosine was detected after 60 min. If a phosphodiesterase was responsible for the cleavage of the diphosphate producing nicotinamide plus 2′-P-adenosine (Fig. 4), a second phosphatase must subsequently hydrolyze the 2′-phosphate to produce adenosine. Other submitochondrial fractions had no significant rates of $NADP^+$ degradation (results not shown). Since the OM fraction metabolised both NAD^+ and $NADP^+$ (Tables 3 and 4) it is possible that the enzymes responsible for NAD^+ breakdown are not completely specific for this coenzyme.

During the long-term incubation of potato tuber mitochondria some of the NAD^+ breakdown products were recovered in the mitochondria and others in the medium (Figs 3*B*, *C*). Given that the NAD^+-hydrolyzing enzymes are localized to the OM, one can conclude that NAD^+ diffuses intact across the inner membrane into the IMS as previously suggested (Neuburger and Douce 1983; Neuburger *et al.* 1985). NAD^+ degradation enzymes in the OM will produce breakdown products in the IMS that can passively cross the OM and leave the mitochondria. It is possible, however, that some metabolites are taken up again across the inner membrane, which could partly explain the appearance of AMP inside the mitochondria after long-term incubation on ice (Fig. 3*B*).

Table 4. Metabolites detected after incubation of $NADP^+$ with outer membranes from potato tuber mitochondria
Conditions as in Table 3, except that incubations were with 100 μM $NADP^+$ instead of NAD^+

Fraction	Metabolite	Amount (pmol mg^{-1}) 0 min	60 min	Rate (pmol mg^{-1} min^{-1})
OM	NMN	0	11 100	+186
	$NADP^+$	76 000	60 000	–266
	NAD^+	770	0	–13
	Adenosine	0	3270	+54

The presence of several enzymes in the OM involved in NAD^+ metabolism has been established, but we do not know their precise identity or their function in the intact cell. The catalytic capacity was about 100 pmol min^{-1} (mg mitochondrial protein)$^{-1}$ since the outer membrane fraction was about 10-fold purified. Assuming that the mitochondria contain 10–15% and the cytosol 45–55% of the total cellular NAD in potato tuber cells, as observed for barley cell protoplasts (Wigge *et al.* 1993), the cytosol contains a total of about 10 nmol NAD per mg of mitochondrial protein. Thus, the OM activity has the capacity to hydrolyze all the cytosolic NAD in 2–3 h depending on its kinetic properties. Therefore, this catabolism needs to be regulated. If NAD^+ metabolites, produced from the action of NAD^+-catabolising OM enzymes, have metabolic roles in the mitochondrial matrix one would expect to find transport mechanisms across the inner membrane for various NAD^+ metabolites. However, such transporters still remain to be identified as do the main sinks for the NAD^+ metabolites in cellular metabolism.

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