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DYNAMIC CHANGES IN THE REDOX LEVEL OF NAD IN POTATO TUBER MITOCHONDRIA OXIDISING MALATE

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Introduction

The respiratory chain of plant mitochondria contains three NAD(P)H dehydrogenases on the matrix surface of the inner membrane: Complex I, which is rotenone- and diphenyleneiodonium (DPI)-sensitive and has a low Km(NADH); ND_{in}(NADH), which is rotenone- and DPI-insensitive and has a high Km(NADH); and ND_{in}(NADPH), which is rotenone-insensitive and DPI-sensitive (Møller and Rasmusson 1998). The activities of these dehydrogenases are dependent on the concentration and reduction levels of NAD and NADP in the matrix. For matrix NAD, there is indirect evidence from three different types of experiments to indicate that NAD^+-malate dehydrogenase (MDH), due to its high activity and the extremely low equilibrium constant of its reaction, has a central role in regulating the reduction level of pyridine nucleotides (Møller and Lin 1986 and references therein): (i) Measurements of oxygen consumption using NAD^+-linked Krebs cycle intermediates as substrates. For instance, when the rate of rotenone-insensitive malate oxidation increased it was concluded that the matrix concentration of NADH had increased. (ii) Measurements of the concentrations of Krebs cycle intermediates, mainly malate and oxaloacetate (OAA). Thus, when the concentration of OAA decreased it was concluded that the reduction level of NAD had increased. (iii) The most direct method was the use of NAD(P)H fluorescence to estimate changes in the amount of NADH during malate oxidation (Neuburger *et al.* 1984).

None of the above methods could quantify the NAD concentration or reduction level in the mitochondrial matrix. With the discovery of a number of enzymes utilising matrix NADP(H) (Møller and Rasmusson 1998 and references therein) the question also arose as to what extent matrix NADP(H) contributed to previous observations.

In the present study we have therefore used extraction and HPLC analysis to quantify the amounts and reduction levels of NAD and NADP in potato tuber mitochondria, under conditions previously studied using indirect methods.

Materials and Methods

Potato tubers (*Solanum tuberosum* L. cv. Bintje) were purchased from local markets. Mitochondria were isolated as described by Struglics *et al.* (1993). Respiration was measured as oxygen consumption in a Rank Brothers oxygen electrode. The temperature was maintained at 15°C to permit measurements at the very high protein concentration (8 mg ml^{-1}) used. The reaction medium contained 0.3 M sucrose, 5 mM MOPS, 5 mM KH_2PO_4, 2.5

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mM $MgCl_2$, 0.01% (w/v) BSA, 1 mM EGTA [to inhibit ND_{ex} activity (Palmer *et al.* 1982)], and 10 mM malate, pH 6.5, 7.2 or 7.5. For measurements conducted at pH 6.5, 0.05 mM coenzyme A was added to optimise conditions for malic enzyme (ME) activity. Glutamate at 10 mM was added to assays carried out at pH 7.2 to aid in removing OAA. For continuous state 3 conditions, 10 units hexokinase, 0.05 mM ADP, 30 mM glucose were added, whereas for state 4 and anaerobiosis, 0.05 mM ADP was added to give an initial state 3 – state 4 cycle. Antimycin A at 5 µM was added to anaerobic incubations to minimise reoxidation of NAD(P)H through the respiratory chain prior to or during extraction.

Samples were taken out from the oxygen electrode for extraction when 40% oxygen had been consumed except for anaerobiosis where the oxygen had been completely depleted by malate oxidation and the incubation left for a further 10 min before sampling. Pyridine nucleotides were extracted as described in Stocchi *et al.* (1985) and the extracts analysed by reversed phase HPLC on a Grom-Sil ODS-O-AB column (5 µm; 125 x 2 mm) using the following program: 0-7 min, 100% buffer A (0.3 M KH_2PO_4, pH 6.0); 7-26 min, 0-50% buffer B (0.1 M KH_2PO_4 containing 10% (v/v) methanol, pH 6.0); 26-27 min, 100%B; 27-28 min, 50% B; 28-30 min 100% A; Flow rate: 0.3 ml min^{-1}. Absorbance was monitored at 259 nm. Recoveries of NAD^+, $NADP^+$, NADH and NADPH were 66-100%.

Mitochondrial protein was measured according to the Lowry method with BSA as standard.

Results and Discussion

The total amount of NAD extracted from potato tuber mitochondria was 1-2 nmol (mg protein)$^{-1}$ and that of NADP was 0.1-0.2 nmol mg^{-1} in agreement with Brinkmann *et al.* (1973) and Wigge *et al.* (1993). In contrast, Roberts *et al.* (1997) found more NADP than NAD in potato tuber mitochondria using ^{31}P-NMR. In all cases, the free nucleotide pool was overestimated, since part of these cofactors are bound to enzymes and/or enzyme complexes within the mitochondrial matrix (Wigge *et al.* 1993). In our experiments, NADPH was always at or below the detection limit (0.02 nmol mg^{-1}), indicating that the reduction level of NADP was below 10%. In the following discussion only the reduction level of NAD will be addressed.

The reduction level of NAD during malate oxidation was investigated under three different conditions: at pH 6.5 where only ME is active, at pH 7.2 in the presence of glutamate where both ME and MDH are active but OAA is removed by transamination, and at pH 7.5, where only MDH is active and no OAA-removing system is present.

In potato tuber mitochondria oxidising malate, NAD was most highly reduced when the reaction had reached anaerobiosis (Fig. 1). However, the levels vary with assay conditions. In the pH 6.5 and 7.2 treatments, NAD was more than 80% reduced, whereas at pH 7.5 the reduction level was below 40%. Thus, at anaerobiosis, the reduction level reflects the thermodynamic equilibria of the NAD^+-reducting reactions, i.e., OAA accumulation, at the different pH.

With increasing pH, lower levels of reduction were also observed in potato mitochondria oxidising malate in state 3 (Fig. 2). At pH 6.5, 47% of the NAD pool was reduced whereas at pH 7.2, the reduction level decreased to 10%. At pH 7.5 no NADH was detectable. This decrease in the reduction state of the NAD pool with increasing pH is again the direct result of an increasing accumulation of OAA (Palmer *et al* 1982, Neuburger *et al.* 1984).

It has previously been demonstrated that the addition of rotenone to potato mitochondria oxidising malate increased the NAD(P)H fluorescence which was interpreted as an increase in the reduction level of the NAD pool (Neuburger *et al.* 1984). In the present study HPLC

analysis revealed increased amounts of NADH above that of state 3 with potato mitochondria oxidising malate in the presence of rotenone at pH 7.2 (Fig. 2). At pH 7.5, the mitochondria were unable to synthesise detectable amounts of NADH even in the presence of rotenone. However, independent of the presence of rotenone, potato mitochondria exposed to a more acidic medium appear to be capable of maintaining a more reduced state of NAD (Fig. 2). This would facilitate the involvement of the ND_{in} (NADH) which has a higher Km for NADH than complex I (Møller and Rasmusson, 1988, Agius *et al* 1988). Under state 2 and state 4 (pH 6.5 and pH 7.2) the same trend was seen in the level of NAD reduction (Figure 1).

In summary, the extraction and quantification of NAD(H) by HPLC analysis confirms previous results obtained by indirect methods: The oxidation of malate in the matrix of plant mitochondria is accompanied by dynamic changes in the reduction level of the NAD pool and these changes are modulated by factors such as the pH, enzyme kinetics, respiratory state and the concentration of pyridine nucleotides (Møller & Lin 1986 and references therein). The low amounts of $NADP^+$ and NADPH detected in potato tuber mitochondria under the conditions studied preventing us from determining to what extent NADPH turnover was involved in malate oxidation as reported by Agius *et al.* (1998).

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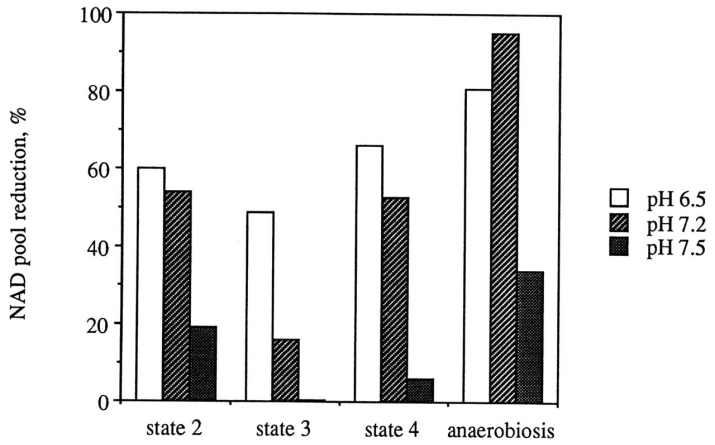

Fig. 1. The reduction level of NAD in potato tuber mitochondria oxidizing malate in different respiratory states at pH 6.5 (with coenzyme A), pH 7.2 (with glutamate) and pH 7.5. The data presented are means of two replicates on one mitochondrial preparation, however, similar results were obtained on another preparation.

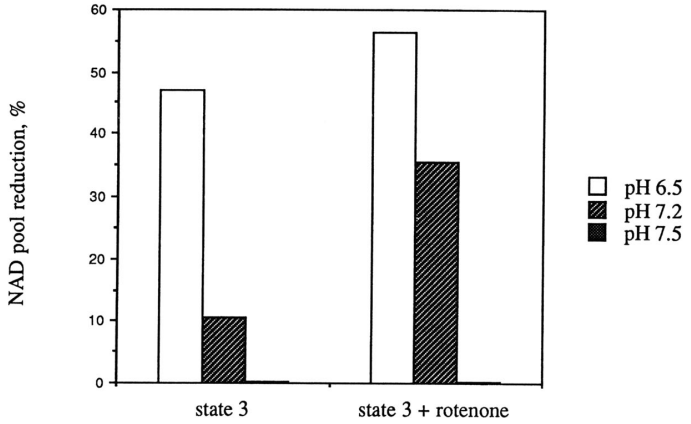

Fig. 2. The reduction level of NAD in potato tuber mitochondria oxidizing malate as affected by rotenone. Conditions as in Fig. 1.

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