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NADP-Utilizing Enzymes in the Matrix of Plant Mitochondria[1]

Allan G. Rasmusson and Ian M. Møller*

Department of Plant Physiology, University of Lund, Box 7007, S-220 07 Lund, Sweden

ABSTRACT

Purified potato tuber (*Solanum tuberosum* L. cv Bintje) mitochondria contain soluble, highly latent NAD$^+$- and NADP$^+$-isocitrate dehydrogenases, NAD$^+$- and NADP$^+$-malate dehydrogenases, as well as an NADPH-specific glutathione reductase (160, 25, 7200, 160, and 16 nanomoles NAD(P)H per minute and milligram protein, respectively). The two isocitrate dehydrogenase activities, but not the two malate dehydrogenase activities, could be separated by ammonium sulfate precipitation. Thus, the NADP$^+$-isocitrate dehydrogenase activity is due to a separate matrix enzyme, whereas the NADP$^+$-malate dehydrogenase activity is probably due to unspecificity of the NAD$^+$-malate dehydrogenase. NADP$^+$-specific isocitrate dehydrogenase had much lower K_ms for NADP$^+$ and isocitrate (5.1 and 10.7 micromolar, respectively) than the NAD$^+$-specific enzyme (101 micromolar for NAD$^+$ and 184 micromolar for isocitrate). A broad activity optimum at pH 7.4 to 9.0 was found for the NADP$^+$-specific isocitrate dehydrogenase whereas the NAD$^+$-specific enzyme had a sharp optimum at pH 7.8. Externally added NADP$^+$ stimulated both isocitrate and malate oxidation by intact mitochondria under conditions where external NADPH oxidation was inhibited. This shows that (a) NADP$^+$ is taken up by the mitochondria across the inner membrane and into the matrix, and (b) NADP$^+$-reducing activities of malate dehydrogenase and the NADP$^+$-specific isocitrate dehydrogenase in the matrix can contribute to electron transport in intact plant mitochondria. The physiological relevance of mitochondrial NADP(H) and soluble NADP(H)-consuming enzymes is discussed in relation to other known mitochondrial NADP(H)-utilizing enzymes.

Plant mitochondria are generally held to utilize only NAD(H) as the reducing equivalent for its respiratory processes, whereas NADP(H) is considered to be associated mainly with the bio- and photosynthetic reactions of the cytoplasm and chloroplasts (10). NADP(H) also takes part in maintenance, as the reduced form is the substrate of GR[2], reducing GSSG to GSH. GSH functions as an antioxidant in the plant cell. In chloroplasts it interacts with dehydroascorbate reductase and ascorbate peroxidase to remove H_2O_2 formed in the light reaction, but in the rest of the cell it is controversial whether GSH is oxidized by that mechanism or by GSH peroxidase when scavenging H_2O_2 (27).

NADPH-specific GR has recently been found and characterized in mitochondria from pea leaves (7). H_2O_2 can be generated by autooxidation of redox components in the mitochondrial electron transport chain by O_2 (9), either directly or through the superoxide dismutase with superoxide radicals as intermediates; GR might participate in the removal of the H_2O_2 formed.

Another NADP(H)-utilizing enzyme, the nicotinamide nucleotide transhydrogenase, has also been reported to be present in plant mitochondria (3). The transhydrogenase in animals is an energetically coupled membrane-bound enzyme catalyzing the conversion of NADH + NADP$^+$ into NAD$^+$ + NADPH and vice versa (12). This reaction can also be accomplished without proton translocation by the mammalian complex I (24) and the two activities are difficult to distinguish.

Mammalian mitochondria are known to contain NADP(H) and to keep it in a much more reduced state than NAD(H). This reduction is thought to be carried out by the energy-linked transhydrogenase mentioned above and an NADP$^+$-ICDH (12). Studies with the NADP$^+$-ICDH inhibitor D-threo-α-methylisocitrate on isocitrate oxidation by intact rat liver mitochondria have shown that a substantial part of the total isocitrate oxidation goes through the NADP$^+$-linked enzyme (26).

To see what possible ways NADPH could be generated in plant mitochondria we studied NADP$^+$-reducing activities in intact, purified potato tuber mitochondria.

MATERIALS AND METHODS

Isolation of Mitochondria

Mitochondria were isolated by a modification of the method of Petit *et al.* (23). Locally purchased potato tubers (*Solanum tuberosum* L. cv Bintje) were homogenized with a juice extractor into a medium (170 mL/kg potatoes) initially containing 0.9 M mannitol, 30 mM Mops, 3 mM EDTA, 25 mM cysteine (pH 7.3), and 0.3% (w/v) BSA. pH was kept constant by drop-wise addition of 8 M KOH. The homogenate was left standing for 5 min allowing the starch to sediment and was then filtered through nylon gauze and centrifuged at 400*g* for 11 min. The supernatant was decanted and centrifuged at 10,000*g* for 23 min. The pellets were resuspended in wash medium (0.3 M mannitol, 10 mM Mops, 1 mM EDTA [pH 7.2], and 0.1% [w/v] BSA) and centrifuged at 10,000*g* for 23 min. These second pellets were also resuspended in wash medium and are referred to as crude mitochondria.

Purification was made on a 4-step Percoll gradient containing (bottom to top) 5 mL 80%, 7.5 mL 40%, 12.5 mL 23%, and 7.5 mL 10% Percoll. The whole gradient contained 0.3 M mannitol, 10 mM Mops (pH 7.2), and 0.1% (w/v) BSA. The gradient was centrifuged at 10,000*g* for 32 min. Bands at the 10/23% interface (broken mitochondria and amyloplasts)

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[2] Abbreviations: GR, glutathione reductase; ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase.

Table I. *Specific Activities and Latencies of Some NADP-Utilizing Enzymes in Intact Purified Potato Mitochondria*

Rates are shown as means ± SD for three independent mitochondrial preparations.

Enzyme	Parameter	Coenzyme	
		NAD(H)	NADP(H)
MDH	Activity[a]	7.2 ± 2.0	0.16 ± 0.04
	Latency, %	93	95
ICDH	Activity[b]	160 ± 40	25 ± 7
	Latency, %	96	93
GR	Activity[b]	Not detected	16 ± 1
	Latency, %		79

[a] μmol min^{-1} mg^{-1}. [b] nmol min^{-1} mg^{-1}.

and at the 23/40% interface (purified mitochondria) were taken out and washed twice by resuspension in wash medium and centrifugation at 12,000*g* for 16 min. The two fractions were finally resuspended in wash medium without BSA and used fresh.

All procedures were carried out at 4°C using precooled angle rotors (JA 14 and JA 20) and a Beckmann J2 21M refrigerated centrifuge.

$(NH_4)_2SO_4$ Precipitation of ICDH

Pure mitochondria were diluted four-fold in 10 mM KH_2PO_4 (pH 7.2), to a protein concentration of approximately 5 mg/mL and were stirred on ice for 25 min. $MgCl_2$ was added to a concentration of 1 mM. This suspension was sonicated 5 × 5 s with 1 min between the bursts with an MSE 150 W Ultrasonic disintegrator set on high power and 16 microns. It was centrifuged at 48,000*g* for 4 min to remove larger membrane fragments. The supernatant was transferred to a series of tubes with varying amounts of $(NH_4)_2SO_4$ and was mixed thoroughly. After 30 min the tubes were centrifuged at 10,000*g* for 10 min and the supernatants separated from the pellets. The pellets were resuspended in 0.4 M $(NH_4)_2SO_4$ and 10 mM KH_2PO_4 (pH 7.2), because initial experiments had shown that the NAD^+-ICDH was very unstable in the phosphate buffer if the $(NH_4)_2SO_4$ was excluded.

Enzyme Assays

Cyt *c* oxidase was measured spectrophotometrically (Aminco DW-2) at 550 nm. The medium consisted of 0.3 M sucrose, 50 mM Tris/acetate (pH 7.2), 45 μM reduced Cyt *c*, and 100 mM KCl when measuring the nonlatent rate; 0.025% (w/v) Triton X-100 was added to measure the total activity.

ICDH activity was measured as production of NAD(P)H at 340 nm in a medium containing 0.3 M sucrose, 50 mM Tes (pH 7.4), 5 mM DL-isocitrate, 1 mM $MgSO_4$, and 200 ng/mL antimycin A unless otherwise indicated. The reaction was started by the addition of 1 mM $NAD(P)^+$ or the sample and 0.025% Triton X-100 was used to solubilize membranes. Latency (%) was calculated as:

$$100 \times ([\text{Rate} + \text{TX}] - ([\text{Rate} - \text{TX}] - [\text{Rate} - \text{NAD(P)}^+]))/[\text{Rate} + \text{TX}]$$

The subtraction of the term [Rate-$NAD(P)^+$] was made to correct for mitochondrial swelling. Kinetic parameters were determined using mitochondria solubilised for 2 min in 0.025% Triton X-100. Assay conditions were as above except for the substrate varied.

GR activity was assayed as NAD(P)H (0.2 mM) consumption in 0.3 M sucrose, 100 mM Tes (pH 7.6), 200 ng/mL antimycin A and 0.5 mM GSSG, but otherwise exactly as in the ICDH assay.

Malate dehydrogenase was measured as described in Møller *et al.* (18).

NADPH, malate, and isocitrate oxidation in intact mitochondria were measured in an O_2-electrode (Rank Brothers, Cambridge, UK) in a medium containing 0.3 M sucrose, 5 mM Mops, 5 mM Pi, and 2.5 mM $MgCl_2$ (pH 7.2). The sample was preincubated with 0.5 mM ATP and 1.0 mM EGTA for 1 min before substrate was added.

Protein was determined by the method of Lowry *et al.* (17), using BSA as standard.

Where nothing else is indicated, the results presented are from representative experiments. Similar results have been obtained on at least two independent preparations.

RESULTS AND DISCUSSION

NADP(H)-Utilizing Enzyme Activities

Purified potato mitochondria contain NADPH-GR and $NADP^+$-dependent MDH and ICDH, the latter two highly latent (Table I). This indicates that they are found in the matrix since the rate at which NAD(P)(H) crosses the inner membrane is several orders of magnitude lower than these enzyme activities (13, 29). The somewhat lower latency of GR is probably an artifact; the nonlatent rate is difficult to determine accurately due to light scattering phenomena. The observation of GR activity in the matrix of purified mitochondria from potato tubers confirms a recent report that GR is found in pea leaf mitochondria (7). Thus, GR may be a normal constituent of plant mitochondria. The activities of ICDH and MDH were much smaller with $NADP^+$ as acceptor

Table II. *Distribution of Enzymes and Markers on the Percoll Gradient*

Parameter	Crude Mitochondria	10/23% Interface[a]	23/40% Interface[b]	Yield[c]
				%
Protein, mg	343	22	105	31
Cyt c oxidase				
Total activity[d]	181	18	128	71
Latency, %	96	80	99	—[e]
NAD^+-ICDH				
Total activity[d]	35	1.3	21	61
Latency, %	99	—	99	—
$NADP^+$-ICDH				
Total activity[d]	5.0	0.23	3.1	63
Latency, %	97	87	93	—

[a] Amyloplast membranes + broken mitochondria. [b] Purified mitochondria. [c] In purified mitochondria compared to total activity applied to gradient. [d] μmol min^{-1}. [e] Not measured.

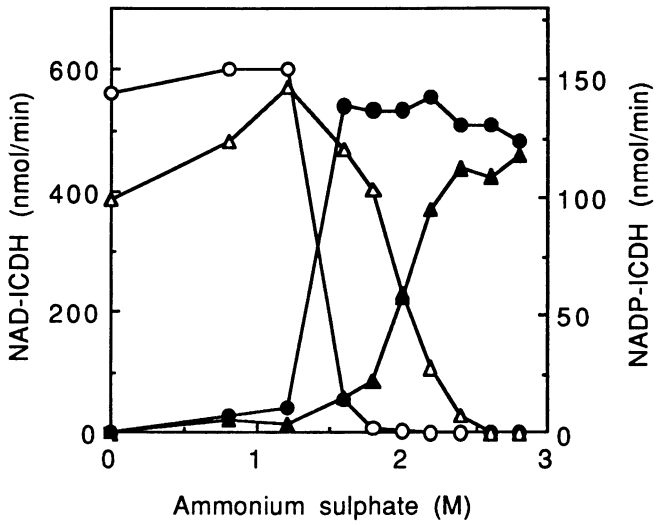

Figure 1. Separation of NAD$^+$-ICDH and NADP$^+$-ICDH by $(NH_4)_2SO_4$ precipitation. $(NH_4)_2SO_4$ precipitation of NAD$^+$(○, ●)- and NADP$^+$(△, ▲)-specific ICDH from a 48,000*g* supernatant after sonication of the mitochondria. Open symbols, supernatant; closed symbols, pellet.

than with NAD$^+$ (16 and 2%, respectively; Table I). NADP$^+$-dependent activities of ICDH and MDH have not been investigated before in purified plant mitochondria.

NADP$^+$ Reduction by ICDH

NADP$^+$-ICDH is found in chloroplasts (8), glyoxysomes, and the cytoplasm (5) of plants. Crude potato mitochondria are heavily contaminated by amyloplast envelopes which band immediately above the purified mitochondria on a Percoll gradient (22). To see whether the activity of NADP$^+$-ICDH in the purified mitochondria could come from contaminating amyloplast membranes, enzyme activities of the crude mitochondria, the yellow 10/23% interface and the purified mitochondria were assayed (Table II). The 10/23% interface contains some broken mitochondria as indicated by the poorly latent Cyt *c* oxidase activity. The NAD$^+$- and NADP$^+$-specific ICDH copurify with each other and with Cyt *c* oxidase on the gradient, and they all have high latencies in the purified mitochondria. Thus, the NADP$^+$-dependent activity is not due to contamination. NADP$^+$-ICDH activity has been reported to band with mitochondria on a sucrose gradient of castor bean endosperm homogenate (5), and some activity has been reported in unpurified potato mitochondria (28), but in neither case was it investigated further.

Having shown that the NADP$^+$-dependent activity was mitochondrial, we wanted to establish whether it was due to an NADP$^+$-specific ICDH or to an unspecific activity of the NAD$^+$-ICDH. Purified mitochondria were sonicated in a high salt medium. This treatment liberates matrix enzymes and produces inside-out submitochondrial particles which do not enclose any matrix components (14, 16). Most of the ICDH was consequently recovered in the matrix fraction (48,000*g* supernatant) after centrifugation (results not shown). The liberated matrix enzymes were exposed to increasing concentrations of $(NH_4)_2SO_4$. The total protein precipitated over a broad range of $(NH_4)_2SO_4$ concentrations with a midpoint at about 1.5 M (results not shown). The enzymes, especially the NAD$^+$-ICDH, were subject to some inactivation although they were kept on ice. By resuspending the pellets in a Pi buffer containing 0.4 M $(NH_4)_2SO_4$ the enzymes were inactivated more slowly than in a Pi buffer alone. Half of the NAD$^+$-ICDH precipitated at about 1.5 M $(NH_4)_2SO_4$, whereas the NADP$^+$-ICDH precipitated at 2.0 M (Fig. 1). Consequently, the 1.6 M pellet contained almost exclusively NAD$^+$-ICDH and the 1.8 M supernatant almost exclusively the NADP$^+$-linked enzyme which was fivefold purified compared to the intact mitochondria (Table III; *cf.* Table I). The separation of the two activities conclusively shows that they are caused by two different enzymes, one NAD$^+$ specific and one NADP$^+$ specific.

NADP$^+$ Reduction by MDH

The fractions from the $(NH_4)_2SO_4$ precipitation experiment were also assayed for NAD(P)$^+$-MDH, but the two activities precipitated identically (results not shown). Thus, the NADP$^+$ reduction is probably due to unspecificity of the NAD$^+$-specific enzyme, although we cannot exclude the presence of a separate NADP$^+$-specific MDH.

Comparison of the Properties of NADP$^+$- and NAD$^+$-ICDH

As the two ICDH activities are due to two different enzymes, each highly specific for its nucleotide (Table III), K_m measurements could be made directly on solubilized mitochondria (Table IV). The NADP$^+$-ICDH had 15- to 20-fold higher affinity for both its substrates than the NAD$^+$-ICDH had, and followed Michaelis-Menten kinetics. Figure 2 shows the specific activities of NAD(P)$^+$-ICDH as a function of the isocitrate concentration. The NAD$^+$-ICDH is cooperative for this substrate. The co-operativity number was determined to be 3.9 using Hill plots. Co-operativity of NAD$^+$-ICDH for isocitrate has previously been reported for pea (4) and potato (28) mitochondria, but with a Hill number of about 2.5.

The K_m determinations for NADP$^+$-ICDH was repeated on the partially purified fraction after further precipitation with $(NH_4)_2SO_4$ and resuspension in 10 mM Pi (pH 7.2). The results

Table III. *Characterization of the Two $(NH_4)_2SO_4$ Fractions Most Enriched in Each of the Two ICDH*

Data are given as means ± SD (number of independent preparations).

Fraction	Protein[a]	Specific Activity	
		NAD$^+$-ICDH	NADP$^+$-ICDH
		nmol min^{-1} mg^{-1}	
1.6 M $(NH_4)_2SO_4$ Pellet	60 ± 15 (4)	233 ± 52 (4)	8 ± 2 (4)
1.8 M $(NH_4)_2SO_4$ Supernatant	22 ± 2 (5)	15 ± 6 (5)	137 ± 9 (5)

[a] % of total (supernatant + pellet) protein.

Table IV. *Kinetic Characterization of the Two ICDH*

The measurements were made on mitochondria solubilized with 0.025% Triton X-100. Eadie-Hofstee plots were used to calculate K_m and V_{max}, which are shown as means ± SD from three (NAD[P]$^+$) or two (isocitrate) independent experiments. The data are from a different set of experiments than those in Table I.

Enzyme	NAD(P)$^+$		DL-Isocitrate	
	K_m	V_{max}	K_m	V_{max}
	μM	nmol min^{-1} mg^{-1}	μM	nmol min^{-1} mg^{-1}
NAD$^+$-ICDH	101 ± 17	241 ± 25	184[a] ± 1	215 ± 13
NADP$^+$-ICDH	5.1 ± 0.8	37 ± 3	10.7 ± 0.8	33 ± 1

[a] Due to the sigmoidicity of the isotherm, $S_{0.5}$ is given.

were almost identical to those presented in Table IV for solubilized mitochondria (results not shown).

The K_m(NAD$^+$) for NAD$^+$-ICDH reported in earlier investigations (4, 28) were higher (approximately, 150 μM) than the 101 μM found by us (Table IV). The reported $S_{0.5}$ for isocitrate was 690 μM for the soluble matrix enzyme in potato at pH 7.8 (28) and 83 μM in pea at pH 7.6 (4). The difference between our isocitrate $S_{0.5}$ of 184 μM and that of (28) may partly be due to different pH in the assay (7.4 in our investigation), since the $S_{0.5}$ increases with higher pH values (4).

The very low K_m of the NADP$^+$-ICDH for isocitrate (see Fig. 2), is interesting since the isocitrate concentration in the mitochondrial matrix is thought to be low. The aconitase equilibrium strongly favors citrate formation—between 10 and 70 times higher concentrations of citrate than isocitrate are found at equilibrium (2). At a low concentration of isocitrate in the matrix the NADP$^+$-ICDH will contribute a substantial part of the total isocitrate oxidation, provided the matrix NADP$^+$ concentration is high enough. The total NAD(H) (NADH + NAD$^+$) concentration in potato mitochondria has been determined to be 1.1 nmol/mg protein (29) and the NADP$^+$ concentration of castor bean endosperm mitochondria can be estimated to about 0.2 nmol/mg protein from the results of Donaldson (5). For spinach leaf mitochondria the NAD$^+$ and NADP$^+$ concentrations have been determined to 0.8 and 0.2 nmol/mg protein, respectively (E Carlenor, personal communication). Assuming a matrix volume of 1μL/mg protein (21) this corresponds to a matrix concentration of about 1 and 0.2 mM for NAD$^+$ and NADP$^+$, respectively. These values should only be taken as guidelines since (a) isolated mitochondria are known to lose NAD$^+$ to the surrounding medium during storage (21) (and probably also during isolation) and (b) NAD$^+$ might be compartmented or bound inside the matrix and not totally available to the enzymes (30). These considerations are probably also valid for NADP$^+$. However, since the K_m(NADP$^+$) for NADP$^+$-ICDH is only 5.1 μM (Table IV) it appears highly probable that sufficient NADP$^+$ is present in the matrix to saturate the enzyme under normal conditions.

The NADP$^+$-ICDH does not show a typical pH optimum but has a maximal activity from 7.4 to 9.0 and declining rates at lower pH values (Fig. 3). The pH optimum of 7.8 for NAD$^+$-ICDH is in agreement with the literature (4, 28). The pH of the cytosol has been determined to be about 7.5 with ^{31}P NMR (25), and the pH in mitochondria is probably slightly higher. This makes NADP$^+$-ICDH a poor candidate for pH regulation. NAD$^+$-ICDH, on the other hand, has a narrow pH optimum, which is shifted downward when the isocitrate concentration is lowered. In addition, it has pH-regulated affinities for its substrates (4).

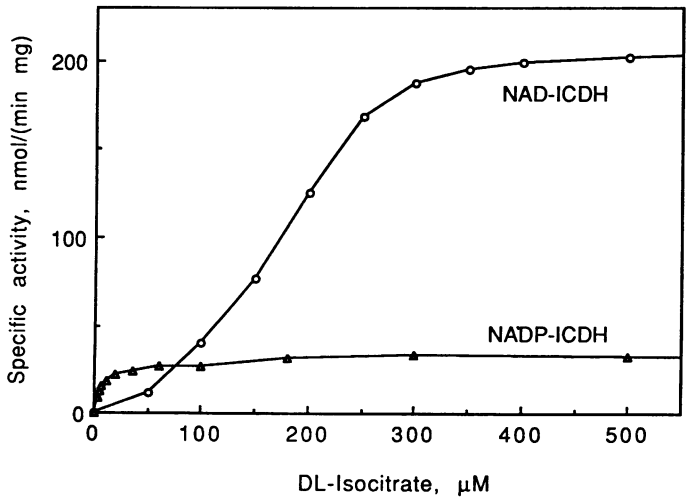

Figure 2. Activity of NAD$^+$- and NADP$^+$-ICDH in solubilized mitochondria as a function of the isocitrate concentration. (For K_m and $S_{0.5}$ values see Table IV).

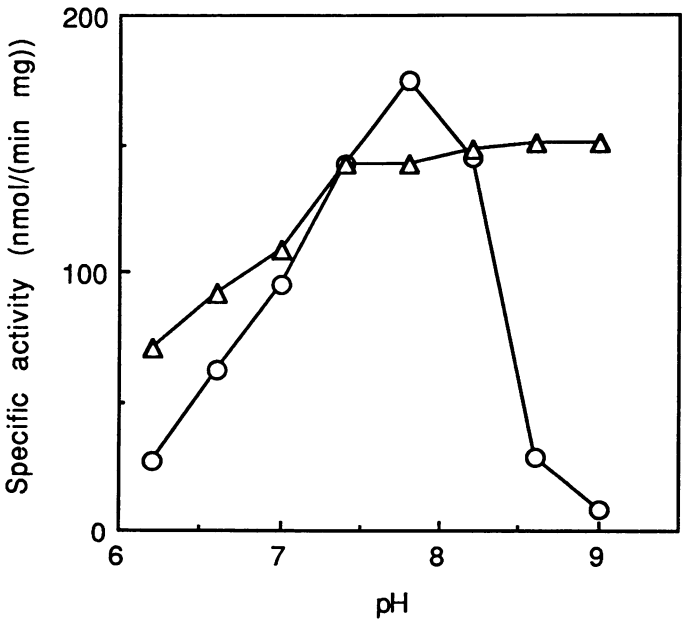

Figure 3. pH curves for NAD$^+$(○)- and NADP$^+$(△)-ICDH in partially purified fractions. The medium was as described in "Materials and Methods" except that the buffer was 50 mM Mes + 50 mM Tes. Similar pH dependence was observed with solubilized mitochondria.

NADP+ Stimulates Malate and Isocitrate Oxidation by Intact Mitochondria

The important question of how externally added NAD^+ stimulated malate oxidation and removed its biphasic appearance was eventually explained by the specific uptake of NAD^+ across the inner mitochondrial membrane (29), and the presence of a rotenone-insensitive, low-affinity NADH dehydrogenase activity on its inner surface (19). Once the NAD^+ is taken up it displaces the MDH equilibrium toward NADH formation and engages the rotenone-resistant, low-affinity NADH dehydrogenase, thereby increasing the O_2-consumption in both states 3 and 4.

Like NAD^+, added $NADP^+$ stimulates malate oxidation by intact mitochondria (Fig. 4). When added in the slow state 4, NAD^+ and $NADP^+$ increase the state 4 rate from 8 nmol O_2 min^{-1} mg^{-1} to 15 and 12 nmol O_2 min^{-1} mg^{-1}, respectively, and the subsequent state 3 rate from 37 nmol O_2 min^{-1} mg^{-1} to 65 and 53 nmol O_2 min^{-1} mg^{-1}, respectively (Fig. 4, C and D). Both NAD^+ and $NADP^+$ take about 5 min to create maximal stimulation (Fig. 4, C and D) while control experiments without any addition shows a constant rate during this time (Fig. 4B). When the mitochondria are preincubated with NAD^+ or $NADP^+$ before the addition of substrate the stimulation is also seen especially during the later state 3/state 4 cycles (*cf.* Fig. 4, E and F with Fig. 4B). The stimulation of malate oxidation is not due to oxidation of NADPH synthesized by nonlatent MDH outside the inner membrane since the external NADPH dehydrogenase has a very low activity under the same conditions (1.0 mM EGTA), even with as much as 1.0 mM NADPH added (Fig. 4A), much more than could possibly be synthesized by external MDH during 5 min (calculations not shown).

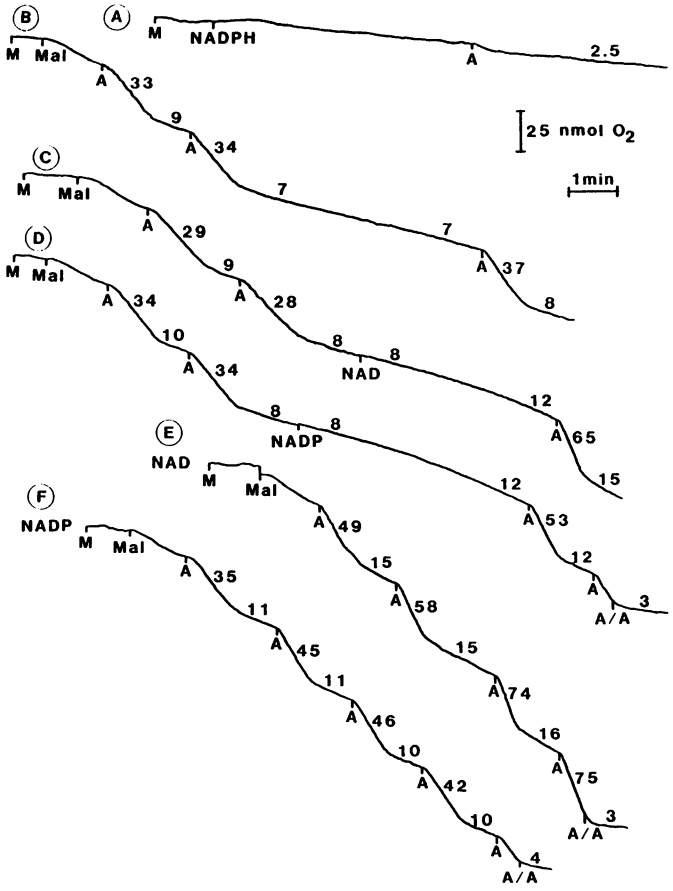

Figure 4. Effect of NAD^+ and $NADP^+$ on malate oxidation by intact mitochondria in the presence of ATP and EGTA. For medium see "Materials and Methods." Additions were (final concentrations in a total volume of 1.0 mL) 1.1 mg/mL mitochondrial protein (M), 1.0 mM NADPH, 0.15 mM ADP (A), Mal, 25 mM malate (Mal), 0.5 mM $NAD(P)^+$, and 200 ng/mL antimycin A (A/A). In E and F, $NAD(P)^+$ were preincubated for 1 min with the mitochondria. The numbers along the tracks are rates in nmol O_2 min^{-1} mg^{-1}.

Table V. *Effect of $NADP^+$ on the State 3 Oxidation of Isocitrate by Intact Mitochondria*

The mitochondria were preincubated with 0.5 mM $NAD(P)^+$. For other conditions see "Materials and Methods."

Addition	O_2 Consumption	
	3 mM isocitrate	10 mM isocitrate
	nmol O_2 min^{-1} mg^{-1}	
Control	12	41
NAD^+	23	51
$NADP^+$	22	48

Table V. Effect of NADP+ on the State 3 Oxidation of Isocitrate by Intact Mitochondria

NADP+ may stimulate malate oxidation through essentially the same mechanism as NAD^+. That means by passing the inner mitochondrial membrane into the matrix and shifting the MDH reaction in the forward direction. The malate-$NADP^+$ oxidoreductase activity of 160 nmol min^{-1} mg^{-1} (Table I) is sufficient to give increased oxygen consumption provided the synthesized NADPH is oxidized by the electron transport chain. This can be achieved by the action of the $NAD(P)^+$ transhydrogenase (3) and/or by the direct oxidation of NADPH by a dehydrogenase on the matrix side of the inner mitochondrial membrane. Oxidation of endogenous NADPH is a well-known ability of the mammalian NADH dehydrogenase (complex I) (11) and the same activity also exists in potato mitochondria (IM Møller, AG Rasmusson, unpublished results).

Similar arguments can be used to explain the $NADP^+$-induced stimulation of isocitrate oxidation by intact mitochondria (Table V) even though we are here dealing with a separate enzyme specific for $NADP^+$. $NADP^+$ stimulates isocitrate oxidation as much as NAD^+ does (Table V) in contrast to what was observed with malate (Fig. 4). This is probably due to two factors: (a) the $NADP^+$-ICDH has a greater activity compared to the NAD^+-ICDH (16%) than the $NADP^+$ activity of MDH has compared to its NAD^+ counterpart (2%) (Table I), and (b) the $NADP^+$-ICDH has a higher affinity for isocitrate than the NAD^+-ICDH has (Table IV; Fig. 2) so that the $NADP^+$-ICDH is operating closer to V_{max}. The stimulation of isocitrate oxidation by $NADP^+$ is not likely to be due to a direct stimulation of $NADP^+$-ICDH which has a very low K_m for $NADP^+$. It is more likely due to an increased concentration of NADPH in the matrix.

The increase in the rate of isocitrate oxidation caused by NAD(P)$^+$ is roughly the same at 3 and 10 mM isocitrate (Table V) while the background activity increases. For NAD$^+$ this is consistent with previously reported results (15).

CONCLUSIONS

Based on our present knowledge we can now outline an NADP(H) cycle in the matrix of potato mitochondria (Fig. 5). NADPH is produced by the NADP$^+$-ICDH and oxidised to NADP$^+$ again by GR which, in turn, reduces GSSG to GSH. The GSH can be used to reduce oxidised sulfhydryl groups in proteins (1) and to participate in the removal of activated oxygen species (27).

The NAD$^+$-ICDH has been considered a possible candidate for intramitochondrial regulation of the TCA cycle (30), as it is the least active enzyme (20), and since isocitrate is oxidized more slowly than other Krebs cycle intermediates (30). NAD$^+$-ICDH can be regulated by a variety of factors, pH, citrate activation, Mg^{2+}, NADH, and substrate concentrations (4). It is possible that the NAD$^+$-linked activity is superimposed, when needed, on a more constant, but lower, NADP$^+$-ICDH activity.

The role of the transhydrogenase (3) is difficult to evaluate as virtually nothing is known about its regulation in plant mitochondria. It might work both as an oxidase and reductase of NADP(H). The enzyme from beef heart, which has been extensively studied, is energetically coupled (12), pumping out a proton across the inner mitochondrial membrane when reducing an NAD$^+$ with an NADPH and letting a proton in when doing the opposite. If this is also true for the plant mitochondrial enzyme it would be a delicate regulator of the reduction level of the matrix NADP(H) and NAD(H).

We do not know the mechanism by which NADP$^+$ is taken up across the inner mitochondrial membrane, but it might be transported on the NAD$^+$ carrier described in (29). However, that would complicate regulation of the mitochondrial NADP(H) concentration. Alternatively, NADP$^+$ is transported on a separate carrier (Fig. 5). Plant mitochondria have previously been shown, directly or indirectly, to take up the following coenzymes: ADP, ATP, NAD$^+$, thiamine pyrophosphate and acetyl-CoA (6). To this list we can now add NADP$^+$.

We conclude that NADP$^+$ can pass the inner membrane of potato mitochondria and become reduced to NADPH by an NADP$^+$-specific ICDH or by MDH, to work as a substrate for NADPH-consuming enzymes like GR.

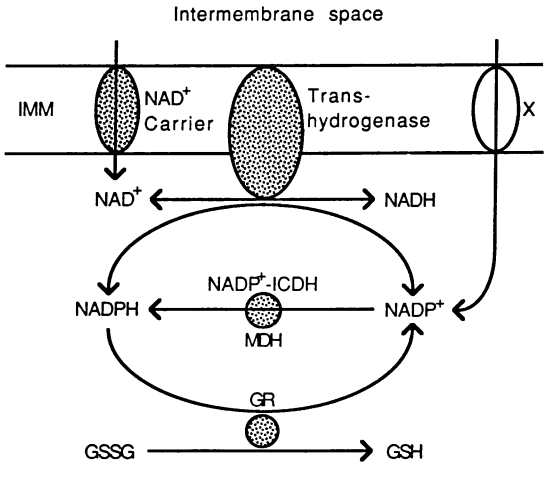

Figure 5. A model for the cycling of NADP(H) in the mitochondrial matrix. NADP$^+$ is transported across the inner mitochondrial membrane into the matrix where it is reduced by MDH, NADP$^+$-ICDH, or the transhydrogenase, subsequently reducing GSSG to GSH with the aid of GR. NADP$^+$ is depicted entering the matrix via a separate carrier 'X,' but it is possible that it is taken up by the NAD$^+$ carrier. Note that the transhydrogenase can work in both directions, both oxidizing and reducing NADP(H). IMM, inner mitochondrial membrane.

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