Plant Physiol. (1993) 102: 579–585

Oxidation of External NAD(P)H by Mitochondria from Taproots and Tissue Cultures of Sugar Beet (*Beta vulgaris*)[1]

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The present study compares the exogenous NAD(P)H oxidation and the membrane potential ($\Delta\psi$) generated in mitochondria isolated from different tissues of an important agricultural crop, sugar beet (*Beta vulgaris*). We observed that mitochondria from taproots, cold-stored taproots, and in vitro-grown tissue cultures contain a functional NADH dehydrogenase, whereas only those isolated from tissue cultures displayed a functional NAD(P)H dehydrogenase. It is interesting that the NADH-dependent $\Delta\psi$ of mitochondria from cold-stored taproots and from tissue cultures was not affected by free Ca^{2+} ions, whereas free Ca^{2+} was required for the mitochondrial NADPH oxidation by in vitro-grown cells and cytosolic NADH oxidation by mitochondria from fresh taproots. A tentative model accounting for the different response to Ca^{2+} ions of the NADH dehydrogenase in mitochondria from cold-stored taproots and tissue cultures of *B. vulgaris* is discussed.

It is well established that mitochondria from yeast cells and plant tissues, unlike those from mammalian cells, have the ability to oxidize externally added NADH (Moller and Lin, 1986). The NADH dehydrogenase catalyzing this activity is insensitive to rotenone, is greatly stimulated by Ca^{2+}, and is not protonmotive (Møller and Lin, 1986). At variance with this general behavior, Day et al. (1976), Rayner and Wiskich (1983), and Soole et al. (1990b) found no NADH oxidation by mitochondria isolated from fresh red beetroots (*Beta vulgaris* cvs Red Globe and Derwent Globe). Conversely, mitochondria isolated from beetroot that had been sliced into discs and "aged" by bubbling in a $CaSO_4$ solution were able to oxidize exogenous NADH (Day et al., 1976). Recently, Fredlund et al. (1991) have shown that mitochondria purified from fresh red beetroots of two different cultivars (Rubria and Nina) can oxidize external NADH, although relatively slowly (25 nmol O_2 min^{-1} mg^{-1} protein, state 3). The rate of NADH oxidation was enhanced (approximately two times) during cold storage of the beetroots for 9 weeks at 10°C, an induction that could be analogous to that caused by washing beetroot slices in $CaSO_4$.

In contrast to NADH oxidation, NADPH oxidation was not induced by cold storage, providing support to the hypothesis that cytosolic NADH and NADPH are oxidized by separate enzymes (Møller and Lin, 1986; Chauveau and Lance, 1991; Fredlund et al., 1991). Whereas the specific induction of an exogenous NADH dehydrogenase in cold-stored and aged beetroot tissues is a well-recognized phenomenon (Day et al., 1976; Arron and Edwards, 1979; Rayner and Wiskich, 1983; Fredlund et al., 1991), the physiological role of the external NAD(P)H dehydrogenase in fresh tissues remains to be established (Arron and Edwards, 1979; Rayner and Wiskick, 1983; Soole et al., 1990a, 1990b; Fredlund et al., 1991; Luethy et al., 1991).

The aim of this work is to contribute to a better knowledge of the external NAD(P)H dehydrogenase in mitochondria isolated from an important agricultural crop, sugar beet (*B. vulgaris*). We have examined the electron transport activities linked to exogenous NAD(P)H oxidation and the resulting $\Delta\psi$'s that are generated in mitochondria from fresh and cold-stored taproots and from in vitro-grown tissue cultures. The results have been interpreted to show that mitochondria from sugar beet plants contain a functional exogenous NADH dehydrogenase, whereas only those isolated from tissue cultures perform both NADH and NADPH dehydrogenase activities. Significantly, the NADH dehydrogenases of cold-stored taproots and tissue cultures were relatively insensitive to Ca^{2+} ions, suggesting that Ca^{2+} per se might not be required for the catalytic activity of this membrane-bound enzyme. These results are discussed in relation to the present models accounting for the mitochondrial oxidation of cytosolic NAD(P)H and the Ca^{2+} dependence of the NAD(P)H dehydrogenase.

MATERIALS AND METHODS

Plant Material

Beta vulgaris L. seeds were kindly provided by Dr. E. Biancardi, Istituto Sperimentale Colture Industriali, Sugar Beet Station, Rovigo-I, where the line MS2 and the hybrid MS2 × 12/77–83 were developed. Sugar beet seeds of the male-sterile MS2 line were sown in paper pots in a peat:sand 1:1 mixture, and seedlings were raised in a greenhouse at 25 to 27°C, 60 to 70% RH until the four-leaf stage. Plants were then artificially vernalized in a cold room for 2 months and

[1] Research work supported by the National Research Council (Consiglio Nazionale delle Ricerche, Bilateral Project Italy-UK) (D.Z. and M.Z.), Ministry of University and Scientific Research and Technology, (D.Z. and M.Z.), and Ministry of Agriculture and Forestry (Sugarbeet National Project) (G.M.).

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Abbreviations: $\Delta\psi$, membrane potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; NAA, naphthaleneacetic acid; P/O ratio, phosphate/oxygen ratio; SHAM, salicyl hydroxamic acid.

subsequently transferred to field conditions. Plants were fertilized once a week with Nitrophoska blue (BASF Agritalia). In the present study, taproots from 1-year-old plants were harvested, washed, and defoliated just prior to use. For comparison, a second genotype (BOT101), possessing N-type normal cytoplasm, was also used.

In Vitro Cultures

Cotyledon sections of the *B. vulgaris* hybrid MS2 × 12/77–83 were used for initial callus induction. Cotyledons were transversely cut into two halves, briefly dipped into absolute ethanol, sterilized for 20 min in 1% (v/v) hypochlorite (180 rpm on a gyrotory shaker), and rinsed five times in double-distilled, sterile water. The explants were then placed on induction medium (PG_oB medium as described by De Greef and Jacobs, 1979) supplemented with 0.7% (w/v) Difco Bacto-Agar, 3% (w/v) Suc, 1 mg/L of NAA, and 0.1 mg/L of kinetin. Explants were incubated at 24°C in the dark for 2 weeks; after this time, about 2 g of white and friable callus were used for suspension culture initiation. Calli were placed in 250-mL flasks containing 50 mL of the callus induction medium (see above) without agar. The flasks were kept in the dark under vigorous continuous shaking (100 rpm) at 24°C. Subcultures were performed weekly by inoculating 5 to 10 mL of the original culture into 40 to 50 mL of freshly prepared medium. The concentration of NAA was reduced to zero over a variable period of 2 to 6 months (auxin-autonomous growth); on the average, three steps of variable duration were needed to lower NAA concentration: from 1 to 0.2 mg/L, from 0.2 to 0.04 mg/L, and from 0.04 to 0.0 mg/L. Once established, the growth of the cultures was characterized by the determination of the dry matter of the whole content of single flasks, after baking at 80°C overnight.

Isolation of Mitochondria

Crude mitochondria from 1-year-old beetroot tissues (270–300 g) were isolated according to Rugolo and Zannoni (1992). Mitochondria were routinely used after a partial purification procedure consisting of a 20-min centrifugation (10,000*g*) through a gradient containing 0.6 M Suc, 10 mM Hepes buffer, pH 7.2, and 1 mM EDTA. The mitochondria were collected at the bottom and washed once by resuspension in 0.3 M mannitol, 2 mM Hepes buffer (pH 7.2). This procedure resulted in approximately 15 to 20 mg of protein. Control experiments were also performed in which crude mitochondria were purified on a discontinuous Percoll gradient consisting of three layers (bottom to top) of 45, 21, and 13.5% (v/v) Percoll. The whole gradient contained 0.5 M Suc, 10 mM Mops buffer, pH 7.2, 0.1% (w/v) BSA, and was centrifuged at 10,000*g* for 35 min; mitochondria were collected at the 21/45% interface and washed once as reported above. This procedure resulted in approximately 5 to 10 mg of mitochondrial protein. However, because the P/O ratios (see below) of Percoll-purified mitochondria were similar to those measured in Suc-purified mitochondria, the latter procedure was routinely used because it was substantially shorter.

The procedure adopted for the isolation of mitochondria from tissue cultures was essentially similar to that described for sugar beet roots except for the following points: (a) the starting material consisted of approximately 80 g of cells (wet weight) grown in two flasks (1 L each) and harvested by means of two wet paper filters; (b) the cell paste was crushed in a chilled (4°C) mortar using the isolation medium as in Rugolo and Zannoni (1992) and then squeezed through four layers of cheesecloth. This procedure was repeated twice. As in the procedure of Rugolo and Zannoni (1992), the final pellet was resuspended in 0.3 M mannitol and 20 mM Hepes buffer (pH 7.2) and was used immediately for further purification as described above for beetroot mitochondria.

The integrity of the outer mitochondrial membrane was assayed by testing the antimycin A sensitivity of the NADH-Cyt *c* oxidoreductase in both intact and osmotically burst mitochondria as previously described (Rugolo and Zannoni, 1992). In Suc-purified preparations, the percentage of mitochondrial membrane integrity was approximately 80%, with P/O ratios of 1.3, 1.5, and 2.3 with succinate, NADH, and pyruvate, respectively. Slightly higher P/O ratios and a percentage of integrity close to 90% were obtained in Percoll-purified mitochondria.

Assays

NAD(P)H dehydrogenase activities were measured as oxygen consumption in a Yellow Springs model 5331 oxygen electrode at 28°C. Experiments were performed using two suspension media: (a) a high-salt medium (0.3 M mannitol, 10 mM Hepes, 0.1% [w/v] BSA, 1 mM $MgCl_2$, 20 mM KCl, 0.5 mM KH_2PO_4, pH 7.2) and (b) a low-salt medium (0.3 M mannitol, 20 mM Hepes buffer, pH 7.2). The free Ca^{2+} concentration of the medium in the presence of the EGTA/Ca^{2+} buffer system was calculated as described by Bers (1982) and corrected for the ionic-strength contribution as in Campbell (1983).

The $\Delta\psi$ was monitored using the lipophilic cation safranine O (Åkerman and Wikström, 1976; Moore and Bonner, 1982) by following the fluorescence signal (excitation at 495 nm, emission at 586 nm), which was quenched with the development of a $\Delta\psi$ in mitochondria (negative inside) upon addition of substrates (Holden and Sze, 1987). All determinations were performed at 28°C in a reaction assay consisting of 0.8 mg of mitochondrial proteins, 0.3 M mannitol, 20 mM Hepes (pH 7.2), and 6 μM safranine. The dye:protein ratio was maintained near 24 nmol/mg protein. To relate the fluorescence quenching of safranine to $\Delta\psi$, a K^+ diffusion potential was induced by adding valinomycin (0.1 μg/mL) to deenergized mitochondria as previously described (Åkerman and Wikström, 1976; Moore and Bonner, 1982). Because it was found that mitochondria from *B. vulgaris* contained a matrix K^+ concentration of about 5 mM (measured by atomic absorption spectroscopy), calibration of the fluorescence quenching as a function of variable K^+ diffusion potentials was performed using rat-liver mitochondria (matrix $[K^+]$ = 125 mM; Åkerman and Wikström, 1976). It should be noted that under our experimental conditions the "zero" diffusion potential as monitored by safranine quenching corresponded to a potential of about 60 mV. Rat-liver mitochondria were kindly provided by Professor G. Lenaz (Department of Biochemistry, Section of Medical Biochemistry, University

Table I. *Respiratory activities of sugar beet (B. vulgaris) taproot mitochondria*

Assays (see "Materials and Methods") were in high-salt medium with final concentrations of 0.3 mg of mitochondrial protein, 100 nM antimycin A (AA), 26 μM rotenone (Rot), 0.5 mM SHAM, 1 μM free Ca^{2+}, 0.2 mM ADP, 0.3 mM NAD(P)H, 5 mM succinate, 5 mM pyruvate, 1.5 mM malate, 1 mM EGTA, and 0.2 mM thiamine pyrophosphate (TPP). Rates are expressed in nmol of O_2 min^{-1} mg^{-1} of protein. Data are the mean values of three preparations.

Experimental Conditions	Rate	Percent of Control
NADH (+Rot)	40.0	100
+ADP	60.0	150
+AA	7.5	18
+AA +SHAM	0.5	1
+EGTA	10.0	25
+EGTA/Ca^{2+}	37.0	93
NADPH (+Rot)	7.5	100
+ADP	9.5	126
+AA	6.5	87
+AA +SHAM	2.5	33
+EGTA	6.5	86
+EGTA/Ca^{2+}	7.0	93
Succinate	37.0	100
+ADP	48.0	130
+AA	12.0	32
+AA +SHAM	0.7	2
Pyruvate (+TPP, +malate)	16.0	100
+ADP	37.0	230
+AA[a]	7.5	20
+Rot[a]	8.5	23
+Rot +AA[a]	7.5	20

[a] Experiments performed in the presence of ADP (1 mM).

of Bologna), and they were isolated by following standard procedures (Johnson and Lardy, 1967).

Protein was determined according to Bradford (1976) with BSA as the standard.

RESULTS

Studies with Mitochondria from Fresh Taproots

As shown in Table I, several exogenously added substrates, except NADPH, were oxidized at a reasonable rate by mitochondria isolated from sugar beetroot. Oxidation of NADH was insensitive to rotenone (26 μM) (not shown) but 75% inhibited by Ca^{2+} depletion (1 mM EGTA). It is interesting that the oxidation of NADH and pyruvate were strongly inhibited by antimycin A (82 and 80%, respectively), whereas succinate oxidation was more resistant (68% inhibition) to the antibiotic; all of these residual activites were, however, 98 to 99% inhibited by 0.5 mM SHAM.

To test the role of the external NAD(P)H dehydrogenase activity as compared to other substrates, e.g. succinate, in generating a $\Delta\psi$, the energized state of sugar beet mitochondria was determined through the fluorescence response of the positively charged dye safranine O (see "Materials and Methods"). As shown in Figure 1A, addition of NADH (0.2 mM) produced a rapid fluorescence quenching of safranine, indicating the formation of a $\Delta\psi$ (140 mV); this quenching was further increased by addition of the protonophore nigericin ($\Delta\psi$ = 160 mV) and rapidly collapsed by the antibiotic antimycin A. A slightly higher ($\Delta\psi$ = 170 mV) safranine quenching was observed upon addition of succinate (Fig. 1D). It is worth noting that, although the succinate-dependent $\Delta\psi$ was almost totally (80%) suppressed by addition of antimycin A, complete reversion of the energization signal was obtained only after addition of the uncoupler FCCP. Trace C of Figure 1 shows that the addition of NADPH did

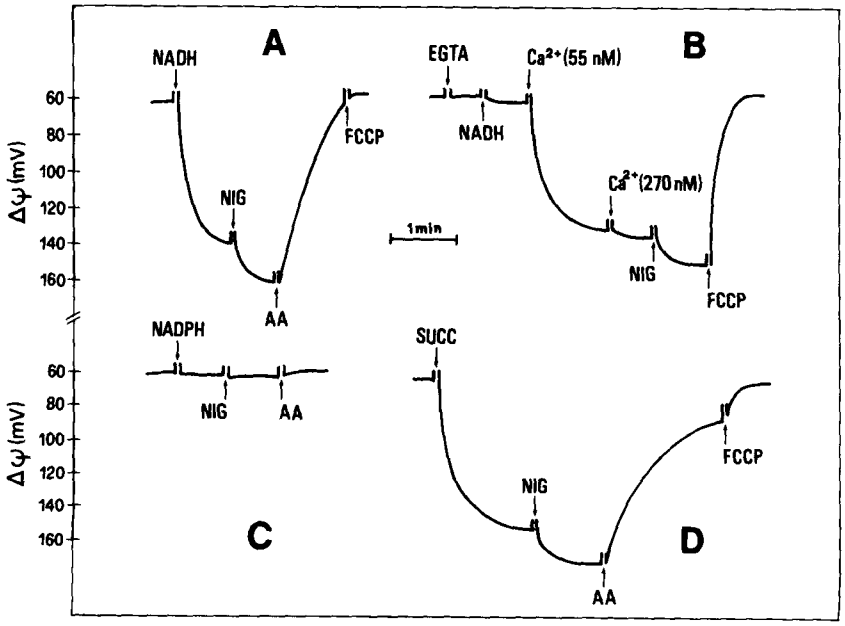

Figure 1. Effects of EGTA, Ca^{2+} ions, antimycin A (AA), nigericin (NIG), and FCCP on the $\Delta\psi$ generated by the oxidation of externally added NADH, NADPH, and succinate (SUCC) in mitochondria from *B. vulgaris* taproots. Experiments were performed with 0.4 mg/mL of mitochondria suspended in 0.3 M mannitol, 20 mM Hepes (pH 7.2). Where indicated, NADH (0.2 mM), nigericin (50 ng/mg protein), antimycin A (50 ng/mg protein), FCCP (360 ng/mg protein), NADPH (0.2 mM), succinate (2.5 mM), and EGTA (1 mM) were added.

Table II. *Respiratory activities of mitochondria isolated from in vitro-grown cells of B. vulgaris (MS2 × 12/77-83)*

Assay conditions, substrates, and inhibitors concentrations are as for Table I. Data are the mean values of four preparations.

Experimental Conditions	Rate	Percent of Control
NADH (+Rot)	75.0	100
+ADP	115.0	152
+AA	37.0	50
+AA +SHAM	3.5	5
+EGTA	35.0	46
+EGTA/Ca^{2+}	75.0	100
NADPH (+Rot)	35.0	100
+ADP	53.0	150
+AA	11.0	30
+AA +SHAM	1.4	4
+EGTA	4.5	12
+EGTA/Ca^{2+}	34.0	97
Succinate	35.0	100
+ADP	49.0	140
+AA	12.5	35
+AA +SHAM	0.7	6
Pyruvate (+TPP, +Malate)	15.5	100
+ADP[a]	31.0	200
+Rot[a]	6.2	20
+AA[a]	15.0	48
+AA +SHAM[a]	4.1	13

[a] Experiments performed in the presence of ADP (1 mM).

not induce a detectable $\Delta\psi$. This latter result was expected due to the low rate of NADPH oxidation (Table I), and is also in line with recent data by Fredlund et al. (1991) indicating that in fresh red beetroot mitochondria, NADPH is oxidized quite slowly (13.8 nmol min^{-1} mg^{-1} protein) and with poor coupling (P/O = 1.0). Figure 1, trace B, also shows that the NADH-dependent $\Delta\psi$ was 98% sensitive to Ca^{2+} ion depletion (1 mM EGTA, Ca^{2+} approximately 1 nM). Notably, a steady-state value of approximately 140 mV was reached after addition of 55 nM free Ca^{2+} ions. Further addition of free Ca^{2+} ions (270 nM final concentration) did not increase the $\Delta\psi$, which was still slightly stimulated (17%) by nigericin.

Studies with Mitochondria from in Vitro Cultures of Sugar Beet Cells

Table II shows that mitochondria isolated from tissue cultures of *B. vulgaris*, similarly to those from fresh taproots, oxidize several substrates with good coupling. It appears, however, that all the activities tested were more resistant (30–50%) to antimycin A than those measured in taproots (18–32%); this residual respiration was 95% inhibited by 0.5 mM SHAM (Table II), suggesting a significant contribution of the alternative oxidase to the respiratory activity of tissue cultures. A second difference between mitochondria from taproots and cell cultures was that, in the latter, a 4.7 times increase of the NADPH oxidative activity (state 3) was observed (Table II).

It is interesting that the oxidation of external NADH in mitochondria from in vitro-grown cells was relatively insensitive (46%) to Ca^{2+} depletion (Table II). This phenomenon was not clearly due to a "Ca^{2+}-cage"-like mechanism, previously proposed to explain the differential effect of Ca^{2+} chelators when added before or after the substrate-activated respiration (Møller et al., 1981; Cowley and Palmer, 1983), because in all the experiments in Table II, mitochondria were preincubated (1 min) with 1 mM EGTA before NADH addition.

As shown in Figure 2A, addition of NADH to mitochondria isolated from tissue cultures induced a significant and rapid quenching of the lipophilic cation safranine O, this signal being equivalent to a potential of about 180 mV ($\Delta\psi$ = 200

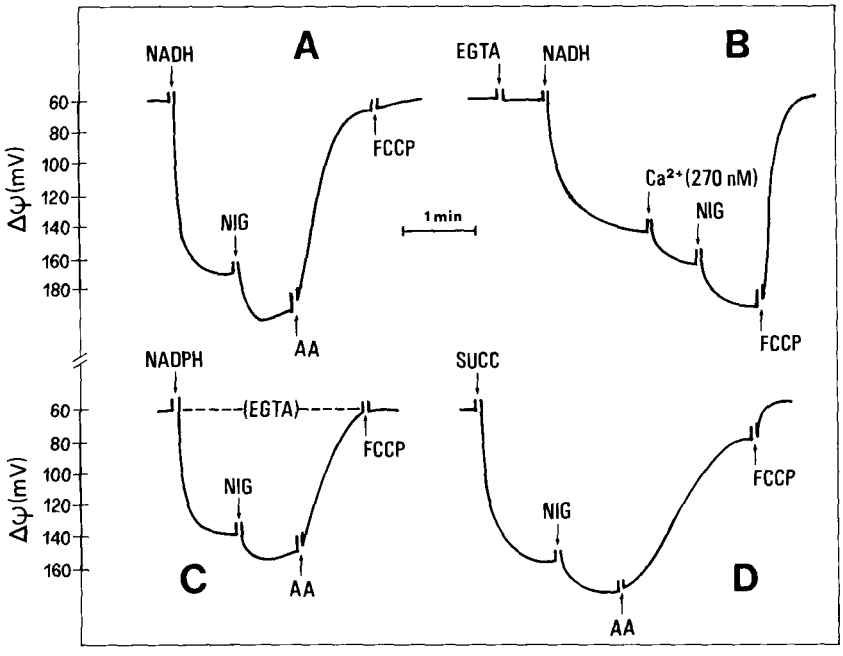

Figure 2. Effects of EGTA, Ca^{2+} ions, antimycin A (AA), nigericin (NIG), and FCCP on the $\Delta\psi$ generated by the oxidation of externally added NADH, NADPH, and succinate (SUCC) in mitochondria isolated from in vitro-grown cells of *B. vulgaris* (MS2 × 12/77-83). Assay conditions were as in Figure 1.

Table III. *Respiratory activities of mitochondria isolated from cold-stored taproot of B. vulgaris*

Assay conditions, subsrates, and inhibitors concentrations are as for Table I. Data are the mean of two preparations.

Experimental Conditions	Rate	Percent of Control
NADH (+Rot)	35.0	100
+ADP	45.5	130
+AA	7.0	20
+AA +SHAM	1.3	4
+EGTA	28.0	80
+EGTA/Ca^{2+}	33.0	94
NADPH (+Rot)	7.5	100
+ADP	10.0	133
+AA	5.5	73
+AA +SHAM	1.5	20
+EGTA	6.5	87
+EGTA/CA^{2+}	7.0	93
Succinate	30.5	100
+ADP	40.5	133
+AA	6.5	21
+AA +SHAM	1.5	5
Pyruvate (+TPP, +Malate)	10.5	100
+ADP	20.0	190
+Rot*	2.5	12
+AA*	4.0	20
+AA +SHAM*	1.0	5

[a] Experiments performed in the presence of ADP (1 mM).

mV in the presence of nigericin). Antimycin A produced a rapid membrane depolarization, supporting the concept that the observed antimycin A-resistant oxidation of exogenous NADH (50%) is not coupled to $\Delta\psi$ generation (Moore and Bonner, 1982).

In contrast to taproot mitochondria, those from in vitro-grown cells performed an NADPH oxidation linked to generation of a $\Delta\psi$ (160 mV, Fig. 2C); this high-energy state was rapidly abolished by antimycin A. As noted in mitochondria from taproots, the succinate-dependent $\Delta\psi$ was not completely collapsed by antimycin A but only by FCCP (Fig. 2D). Remarkably, Ca^{2+} depletion (incubation with 1 mM EGTA) had only a modest effect on the NADH-dependent $\Delta\psi$ (160 mV, Fig. 2B), which was, however, increased to some extent by subsequent additions of free Ca^{2+} ions (270 nM) and by nigericin. In contrast, the $\Delta\psi$ generated by NADPH was drastically inhibited by EGTA (Fig. 2C, dashed line).

Studies with Mitochondria from Cold-Stored Sugar Beet Taproots

It has recently been reported (Fredlund et al., 1991) that the NADH dehydrogenase activity of mitochondria from red beetroots is enhanced during cold storage. To test the effect of aging on sugar beet taproots, a series of experiments was performed in mitochondria isolated from 1-year-old taproots kept in moist sand (at 4°C) for 15 weeks. At variance with Fredlund et al. (1991), the results in Table III show that the respiratory rates of mitochondria from cold-stored taproots were quite similar to those measured in fresh tissues, i.e. (a) the cold storage period did not affect significantly the NADH oxidation rate; and (b) the antimycin A resistance of respiration with both NADH and succinate was only 20% of controls, indicating that mitochondria from taproots (fresh and cold stored) contain low levels of alternative oxidase. However, a major difference between fresh and cold-stored taproots was indeed observed; in the latter case, the NADH oxidation was only 20% inhibited by Ca^{2+} depletion (cf. Tables I and III).

Similarly to mitochondria from fresh taproots, those isolated from cold-stored tissues were able to create a large $\Delta\psi$ (160 mV) with both NADH and/or succinate but not with NADPH (Fig. 3, traces A, C, and D). However, as in the case of tissue cultures, the NADH-dependent $\Delta\psi$ was insensitive

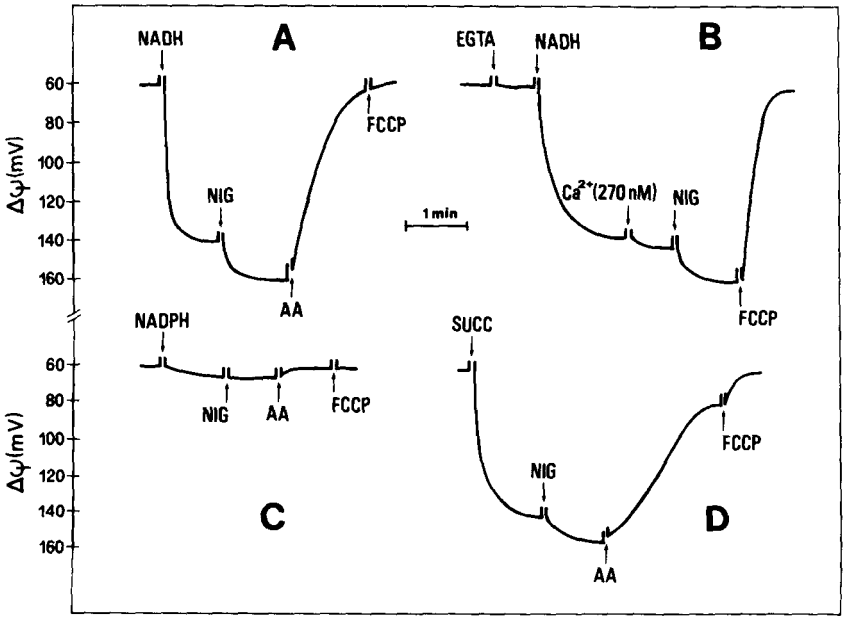

Figure 3. Effects of EGTA, Ca^{2+} ions, antimycin A (AA), nigericin (NIG), and FCCP on the $\Delta\psi$ generated by the oxidation of NADH, NADPH, and succinate (SUCC) in mitochondria from cold-stored taproots. Assay conditions were as in Figure 1.

to Ca^{2+} depletion (trace B). In this respect, the NADH oxidation by cold-stored taproot mitochondria is therefore different from that of fresh tissues.

DISCUSSION AND CONCLUSIONS

Our results concerning electron transport and generation of a $\Delta\psi$ through the oxidation of exogenously added NAD(P)H in mitochondria from *B. vulgaris* can be summarized as follows: (a) mitochondria isolated from fresh taproots of sugar beet plants readily oxidize exogenous NADH (60 nmol min^{-1} mg^{-1} protein) with the generation of a $\Delta\psi$ (160 mV), but they can hardly oxidize NADPH; (b) mitochondria from tissue cultures show good rates of NADH- and NADPH-dependent respiration (115 and 53 nmol min^{-1} mg^{-1} protein, respectively) coupled to generation of a high $\Delta\psi$ (180–200 mV); (c) mitochondria from cold-stored taproots contain a functional, external NADH dehydrogenase (40 nmol min^{-1} mg^{-1} protein; $\Delta\psi$ = 160 mV) but, as in fresh tissues, the oxidation of exogenous NADPH is barely detectable.

It is apparent that results in (a) are different from those of Day et al. (1976), Rayner and Wiskich (1983), and Soole et al. (1990b) with fresh red beetroot mitochondria (cvs Red Globe and Derwent Globe), in which no NADH oxidation was found; our data are also qualitatively and quantitatively discordant with those of Fredlund et al. (1991) with two different cultivars of red beetroots (Rubria and Nina), in which both NADH and NADPH were oxidized, although relatively slowly (12.5 and 18 nmol min^{-1} mg^{-1} protein, respectively). Thus, in contrast to previous equivocal results, the present study unequivocally demonstrates that the external NADH dehydrogenase of mitochondria from fresh taproots of sugar beet plants might be of physiological importance.

As summarized in (b) above, we observed that mitochondria isolated from tissue cultures of *B. vulgaris* are endowed with a functional external NAD(P)H dehydrogenase. In this respect, it should be pointed out that both respiratory activities and related $\Delta\psi$'s were determined in two genotypes (MS2 × 12/77–83 and BOT101) differing markedly not only in nuclear genetic background, but also in the structure and organization of the mitochondrial DNA. Indeed, whereas MS2 × 12/77–83, being a hybrid, has sterile, or so called "S-type," cytoplasm, BOT101 is a maintainer line and therefore contains "N-type," normal cytoplasm. However, no substantial differences in terms of electron transport rates, sensitivities to inhibitors, and energy coupling were observed between the two genotypes (data not shown).

The induction of the NADPH dehydrogenase activity (4.7 times) during in vitro growth of *B. vulgaris* cells might be analogous to induction by the mechanism previously observed in red beetroots upon wounding (Day et al., 1976; Arron and Edwards, 1979) or cold storage (Fredlund et al., 1991), with the significant difference that in these latter cases, NADH, but not NADPH, oxidation was enhanced. The immediate conclusion derived from the present results is that, at least in mitochondria of *B. vulgaris*, the oxidation of cytosolic NADH and NADPH (when present) are catalyzed by separate, specific enzymes. This conclusion, however, is essentially based on measurements of electron transport activities and does not imply a reliable molecular model taking into account the Ca^{2+} response of the NAD(P)H dehydrogenase(s).

The Ca^{2+} dependence of the external NAD(P)H dehydrogenase, which varies among species (Moore and Åkerman, 1982; Nash and Wiskich, 1983; Rugolo and Zannoni, 1992) and tissues of the same species (this work) both in vivo (mitochondria) and in vitro (solubilized enzymes) (Cottingham and Moore, 1984; Cook and Cammack, 1984, 1985; Chauveau and Lance, 1991; Luethy et al., 1991, 1992), has tentatively been explained in several ways, such as (a) conformational change of the enzyme; (b) presence of a membrane-bound Ca^{2+}-regulatory protein closely associated with the enzyme; and/or (c) a differential Ca^{2+}-induced interaction between the enzyme and the ubiquinone pool (Møller and Lin, 1986; Soole et al., 1990b).

Here we report for the first time that in mitochondria from tissue cultures, the NADPH oxidation and the corresponding $\Delta\psi$, but not NADH respiration and its $\Delta\psi$, are fully sensitive to EGTA (Fig. 2). Conversely, in fresh taproots the NADH oxidation and its $\Delta\psi$ are strongly sensitive to Ca^{2+} depletion (Fig. 1), whereas in cold-stored taproots both $\Delta\psi$ and electron flow linked to NADH consumption are largely insensitive to EGTA (Table III and Fig. 3). This suggests that the electron transport models proposed earlier for mitochondrial oxidation of cytosolic NAD(P)H are incomplete (Møller and Lin, 1986).

A more complex proposal, based on the possibility that the NADPH oxidation might derive from some nonspecific catalysis by a single NAD(P)H dehydrogenase (polypeptide), whereas the NADH-dependent respiration might result from the action of multiple NADH dehydrogenases, has recently been formulated (Luethy et al., 1991, 1992). Indeed, several polypeptides (molecular masses of 55, 32, and 42 kD) showing NADH dehydrogenase activity were solubilized from "aged" red beetroot mitochondria (Luethy et al., 1991). It is interesting that although the 55-kD polypeptide was associated with NADH oxidation only, the 42-kD protein was involved in NAD(P)H oxidation. With these results in mind, the present observation that in sugar beet mitochondria there seems to be a tight correlation between EGTA sensitivity of electron transport and EGTA sensitivity of the $\Delta\psi$ with NADPH as substrate but not with NADH might suggest that the NAD(P)H oxidase activity of mitochondria from tissue cultures would be catalyzed by the 42-kD NAD(P)H dehydrogenase (polypeptide).

This proposal, when extended to mitochondria from cold-stored taproots, in which the NADPH oxidation is basically absent but high levels (80%) of Ca^{2+}-insensitive, NADH-dependent respiration and energization are present (Table III and Fig. 3), would imply that there must be a progressive loss during the cold storage period of the catalytic properties of the 42-kD polypeptide (Ca^{2+} sensitive?) with a parallel induction of a Ca^{2+}-insensitive polypeptide (55 kD?). If this hypothesis is true, one may conclude that in intact systems the amount of EGTA-insensitive NADH oxidation would reflect the amount of the 55-kD protein. Recently, the induction of the NADH dehydrogenase activity upon aging of red beet taproots has tentatively been correlated with the appearance of specific polypeptide(s) (Fredlund et al., 1991).

Unfortunately, activity staining of nondenaturating polyacrylamide gels with NADH and nitro blue tetrazolium did not show differences in the banding pattern between mitochondria isolated from fresh and "aged" taproots. It is hoped that further experiments aiming to define the molecular nature of the external NAD(P)H dehydrogenase complex(es) along with its Ca^{2+} dependence will be able to correlate functional and structural studies.

Received December 14, 1992; accepted March 1, 1993.

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