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Two Separate Transhydrogenase Activities Are Present in Plant Mitochondria

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Inside-out submitochondrial particles from both potato tubers and pea leaves catalyze the transfer of hydride equivalents from NADPH to NAD^+ as monitored with a substrate-regenerating system. The NAD^+ analogue acetylpyridine adenine dinucleotide is also reduced by NADPH and incomplete inhibition by the complex I inhibitor diphenyleneiodonium (DPI) indicates that two enzymes are involved in this reaction. Gel-filtration chromatography of solubilized mitochondrial membrane complexes confirms that the DPI-sensitive TH activity is due to NADH–ubiquinone oxidoreductase (EC 1.6.5.3, complex I), whereas the DPI-insensitive activity is due to a separate enzyme eluting around 220 kDa. The DPI-insensitive TH activity is specific for the 4B proton on NADH, whereas there is no indication of a 4A-specific activity characteristic of a mammalian-type energy-linked TH. The DPI-insensitive TH may be similar to the soluble type of transhydrogenase found in, e.g., *Pseudomonas*. The presence of non-energy-linked TH activities directly coupling the matrix NAD(H) and NADP(H) pools will have important consequences for the regulation of NADP-linked processes in plant mitochondria. © 1999 Academic Press

The energy-linked pyridine nucleotide transhydrogenase (EC 1.6.1.1) (H^+-TH) is located in energy-transducing membranes in animal mitochondria and certain heterotrophic and photosynthetic bacteria. The enzyme catalyzes the reversible and stereospecific transfer of hydride equivalents between the 4A position of NAD(H) and the 4B position of NADP(H) with the concomitant translocation of protons across the membrane (1) according to the reaction

$$nH^+_{out} + NADP^+ + NADH \rightleftharpoons NADPH + NAD^+ + nH^+_{in},$$

where nH^+_{out} and nH^+_{in} denote the number of protons translocated vectorially from the cytosol ("out") to the mitochondrial matrix ("in"), respectively. The electrochemical proton potential, $\Delta\mu_{H+}$, drives the reaction from left to right in the intact mitochondrion/bacterial cell (1).

Some heterotrophic bacteria, e.g., *Pseudomonas fluorescens* and *Escherichia coli*, possess a different type of transhydrogenase, a soluble non-energy-linked flavoprotein (2, 3). It transfers the 4B hydrogen from both NADPH and NADH through a ping-pong bi–bi reaction mechanism (4) and is remarkable for the formation of large polymers (5). There is no significant amino acid sequence similarity between the soluble transhydrogenase and the membrane-bound transhydrogenase from *E. coli* (5).

The presence of H^+-TH in plant mitochondria has been suggested (6) but not unambiguously verified. It is still therefore unclear whether plant mitochondria contain a transhydrogenase, and whether it is energy-linked. Plant mitochondria contain the standard set of respiratory complexes, including the H^+-pumping NADH:ubiquinone oxidoreductase or complex I (EC 1.6.5.3). In addition, plant mitochondria contain four NAD(P)H dehydrogenases and the alternative oxidase, none of them H^+-pumping, which provide the plant cell with more metabolic flexibility than a mammalian cell (7). The additional NAD(P)H dehydrogenases are distinguished by their insensitivity to rotenone, the most commonly used complex I inhibitor. Diphenyleneiodonium (DPI), which inhibits complex I uncompetitively by binding on the substrate side of the Fe-S clusters (8), also inhibits the rotenone-insensitive oxidation of matrix NADPH, but not matrix NADH (9, 10).

Abbreviations used: $APAD^+$, 3-acetylpyridine adenine dinucleotide; DPI, diphenyleneiodonium; H^+-TH, energy-linked transhydrogenase; ICDH, isocitrate dehydrogenase; LipDH, dihydrolipoamide dehydrogenase; NR, nitrate reductase; PLM, pea leaf mitochondria; POM, potato tuber mitochondria; SMP, inside-out submitochondrial particles; TH, transhydrogenase.

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In plants, the proton-translocating complex I appears to have properties similar to those of its counterparts in *Neurospora crassa* and bovine heart (11). It is probably the major enzyme involved in the oxidation of NADH because of its low K_m for NADH; its K_m for NADPH is very high and its contribution to NADP(H) turnover uncertain (12–14).

Transhydrogenase activities of the T-D (NADPH to NAD^+) and D-D (NADH to NAD^+) types are also found in mammalian complex I preparations. The T-D transhydrogenation was, however, reevaluated to be caused by contamination with the energy-linked mitochondrial transhydrogenase (15, 16). The D–D transhydrogenase activity has generally been regarded as the reversible first step of the normal NADH oxidation pathway (17–19).

Since NAD(P)H dehydrogenases may carry out transhydrogenase-like activities *in vitro,* we have investigated whether the NADPH–$APAD^+$ activity reported for plant SMP (6) are due to side activities of NAD(P)H dehydrogenases and used a stereospecificity assay to check whether an H^+-TH is involved.

MATERIALS AND METHODS

Mitochondria and inside-out submitochondrial particles (SMP) were isolated from potato tubers (*Solanum tuberosum* L. cv. Bintje) (20, 21) and from 14-days old green pea leaves (*Pisum sativum* L. cv. Oregon sugar) (22, 23) and stored at −80°C in the presence of 5% (v/v) dimethyl sulfoxide.

The thawed SMP were diluted to a final concentration of 1 mg protein/ml in 20 mM Tris–HCl, pH 7.5, 5 mM EDTA, homogenized in a Dounce homogenizer and centrifuged at 100,000*g* for 20 min. The membrane pellet was washed once by the same procedure. The membrane proteins were solubilized by stirring for 30 min on ice in 20 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, pH 7.5 containing 5% (w/v) sucrose and 3% dodecyl maltoside (PLM-SMP) or 4% Triton X-100 (POM-SMP) at 8 mg protein/ml (24, 25). Solubilized proteins were centrifuged at 100,000*g* for 30 min and the supernatant was loaded onto a fast protein liquid chromatography column (Superose 6 HR10/30, Pharmacia Biotech). Proteins were eluted in 20 mM Mops, pH 7.2, 0.5% (w/v) Chaps and 150 mM NaCl at 0.3 ml/min and into 0.5-ml fractions. All the steps were carried out at 4°C.

NADH:ferricyanide oxidoreductase activity in SMP and fractions after gel filtration was monitored at 420 nm. The assay buffer contained 50 mM Tris–HCl, pH 7.2, 50 mM NaCl, 0.2 mM NADH and 1 mM $K_3Fe(CN)_6$ (24).

NAD^+ reduction by NADPH was determined as absorbance change at 340 nm in the presence of $NADP^+$-ICDH and isocitrate as an NADPH-regenerating system (26). The reaction medium contained 0.25 M mannitol, 80 mM Mops–KOH, pH 7.0, 2.5 mM $MgCl_2$, 40 mM KH_2PO_4, 0.02% (w/v) BSA, 2.5 mM threo-D-isocitrate, 1 U $NADP^+$-ICDH, 0.2 mM NADPH and 1 mM NAD^+. The rate of NADPH regeneration exceeded that of the transhydrogenase reaction at least 10-fold and was not rate limiting. A cocktail of inhibitors was added to avoid NAD(P)H oxidation through the respiratory chain: 0.8 μM antimycin A and/or 1 mM KCN, 20 μM rotenone, and 1 mM EGTA (27). One μM carbonyl cyanide *p*-(trifluoromethoxy) phenyl-hydrazone or 0.04% (w/v) Triton X-100 was added and 5 μM DPI was present where indicated.

$APAD^+$ reduction was monitored at 375 minus 420 nm or 366 minus 436 nm using a dual wavelength Shimadzu UV-3000 spectrophotometer (26). The reaction medium contained 50 mM Mes, pH 6.3, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 0.02% (w/v) BSA, 1 mM EDTA, 0.05% (w/v) Brij-35, 0.2 mM NAD(P)H, and 0.2 mM $APAD^+$. For SMP the same inhibitors were used as described above.

The stereospecificity of the TH activities was determined at pH 6.3 by NADPH-dependent reduction of (4-^{3}H)NAD^+ (26, 28) in the absence or presence of 5 μM DPI (primary incubations). After 10 min the primary incubation was stopped by filtration and the stereospecific position of ^{3}H in the product ((4A- or 4B-^{3}H)NADH) was determined in secondary incubations by oxidation with 10 mM $K_3Fe(CN)_6$ using NR (EC 1.6.6.1; 4A-specific) or LipDH (EC 1.8.1.4; 4B-specific) to release ^{3}H as ^3H_2O from 4A-^{3}H-NADH and/or 4B-^{3}H-NADH formed. A control secondary incubation with only ferricyanide, but no NADH-oxidizing enzyme, was also included (see Fig. 2). The activities of NR and LipDH were not affected by the DPI present in some primary incubations and carried over into the secondary incubations (not shown). Labeled end products, ^3H_2O and (4-^{3}H)NAD^+ (as well as residual (4-^{3}H)NADH), were separated on Sephadex G-10 gel-filtration column as described (28).

Protein analysis was performed by SDS–PAGE (29) and immunoblotting and incubation of blots with antibodies against the 78 kDa subunit of *Neurospora crassa* complex I (kind gift of Dr. H. Weiss, Düsseldorf, Germany) as described in (30).

Protein was determined with BSA as a standard (31). In the case of PLM this was corrected for the contribution by thylakoids (<5%) by assuming a thylakoid protein to chlorophyll ratio of 7 (32). Chlorophyll was determined as described (33). Protein concentrations in the gel filtration were estimated using a Micro Assay kit (Bio-Rad) with BSA as a standard.

TABLE I

Transhydrogenase Activities in SMP from Potato Tuber and Pea Leaf Mitochondria

Reaction	Rate (nmol min^{-1} mg $protein^{-1}$)[a] Control	+5 μM DPI[b]
Potato tuber		
NADPH → $APAD^+$[c]	105	10.8
NADH → $APAD^+$[d]	132	8.6
Deamino-NADPH → $APAD^+$[e]	195	15.6
NADPH → NAD^+[f]	13.1	9.2
Pea leaf		
NADPH → $APAD^+$[c]	193	27.4
Deamino-NADPH → $APAD^+$[e]	113	33.2
NADPH → NAD^+[f]	9.4	10.0

[a] Each value is an average of 2–4 measurements on a representative preparation of SMP.

[b] Incubation of the samples with 5 μM DPI for 7 min prior to the reaction was used to reach full inhibition.

[c,d,e] Concentrations of NADPH, $APAD^+$, NADH, deamino-NADPH, and assay conditions were as described under Materials and Methods.

[f] Measured with $NADP^+$-ICDH as an NADPH-regenerating system as described under Materials and Methods.

RESULTS

Both potato tuber and pea leaf SMP carry out transhydrogenase reactions. SMP from both potato tubers and pea leaves, which have the inner, matrix surface of the inner mitochondrial membrane facing the medium

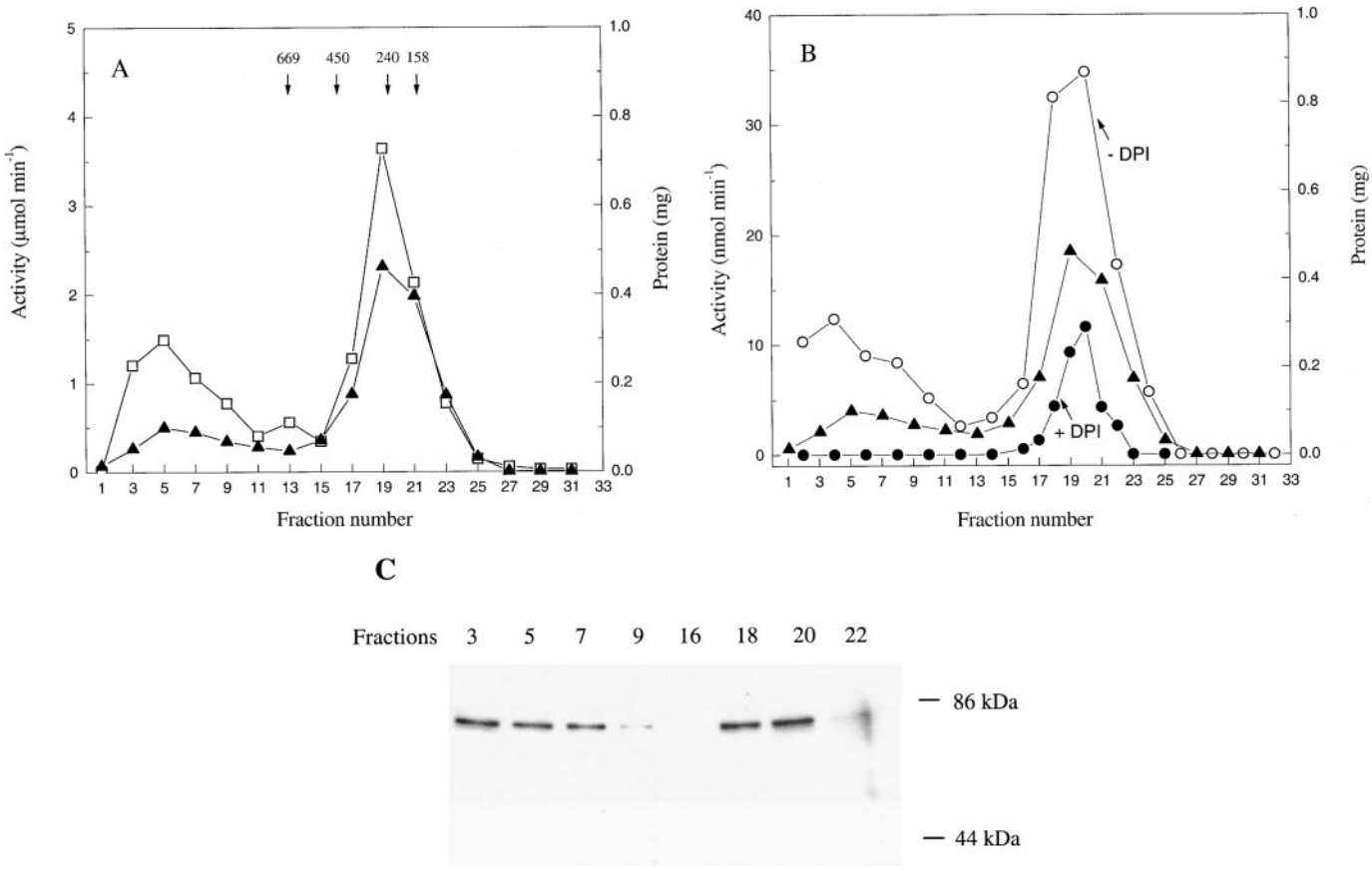


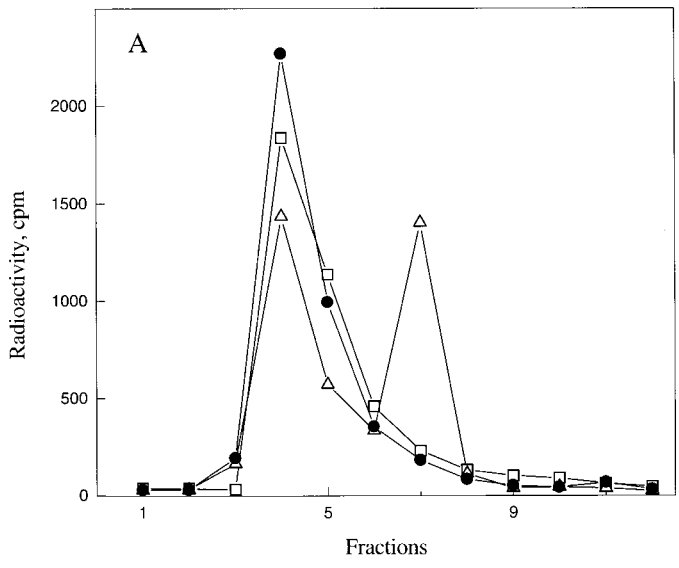
FIG. 1. Gel filtration separation of solubilized PLM-SMP. (A and B) Dehydrogenase and transhydrogenase reactions in the fractions; (C) Western blot analysis with antibodies against the 78-kDa subunit of *N. crassa* complex I. Arrows (in A) show the molecular mass calibration of the column in kDa; open squares, NADH-ferricyanide reductase activity; open circles, NADPH–$APAD^+$ transhydrogenase reaction in the absence of DPI; filled circles, transhydrogenase reaction in the presence of 5 μM DPI; filled triangles, protein content in each 0.5-ml fraction.

(10, 21) catalyzed the transfer of hydride equivalents from NAD(P)H to the NAD^+ analogue, $APAD^+$ (Table I). Reduction of $APAD^+$ by NADPH (T-D activity), NADH (D-D activity) as well as the NADPH analogue deamino-NADPH were strongly, but not completely, inhibited by DPI. This strongly indicates that complex I contributed to all three activities. NAD^+ was also reduced by NADPH (Table I) as monitored with an NADPH-regenerating system. Addition of 5 μM DPI did not inhibit this TH activity indicating that complex I was not involved under the conditions used. In all cases the DPI-insensitive activity was 10-30 nmol min^{-1} mg^{-1} (Table I).

These results indicate that two T-D transhydrogenase activities were present on the inner surface of the inner membrane of both POM and PLM—one DPI-sensitive probably caused by complex I, the other DPI-insensitive.

Size separation of transhydrogenase activities. To identify the DPI-sensitive and -insensitive activities, washed PLM-SMP were solubilized, the enzyme complexes separated by gel filtration and NADH-ferricyanide reductase activity and NADPH-$APAD^+$ transhydrogenase activity measured, the latter in the presence and absence of 5 μM DPI. Two peaks of both ferricyanide and DPI-sensitive $APAD^+$ reduction could be distinguished (Fig. 1), one eluting at a size above 700 kDa (fractions 3–8) and the second peak between 350 and 100 kDa (fractions 15-25). DPI-insensitive transhydrogenase activity was only found in the second peak where most of the protein also eluted (Fig.

FIG. 2. Stereospecificity of PLM-SMP transhydrogenases. (A and B) PLM-SMP assayed in the absence and presence of 5 μM DPI in the primary incubations, respectively; (C) fraction 4 from gel filtration assayed in the absence of DPI; (D and E) fraction 20 assayed in the absence and presence of 5 μM DPI, respectively. Open squares, control secondary incubation in the absence of an oxidizing enzyme; open triangles, oxidation of product by NR (4A-specific) in the secondary incubation; closed circles, oxidation of product by LipDH (4B-specific) in the secondary incubation.


A
Radioactivity, cpm
0
500
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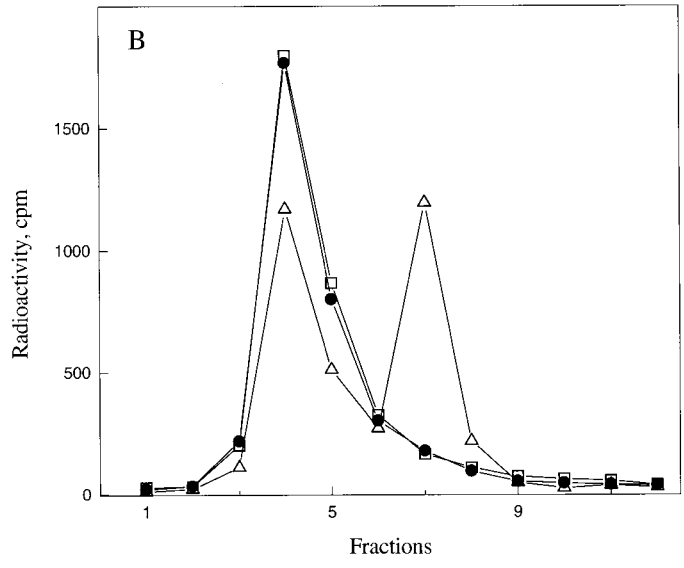
1500
2000
1
5
9
Fractions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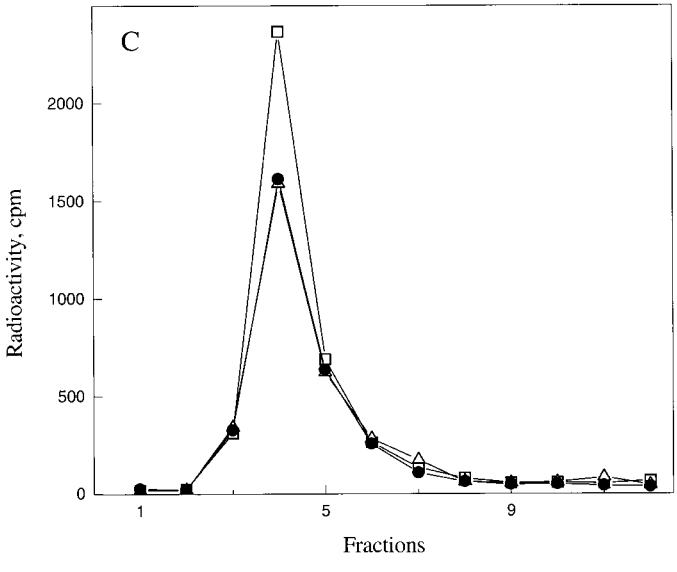
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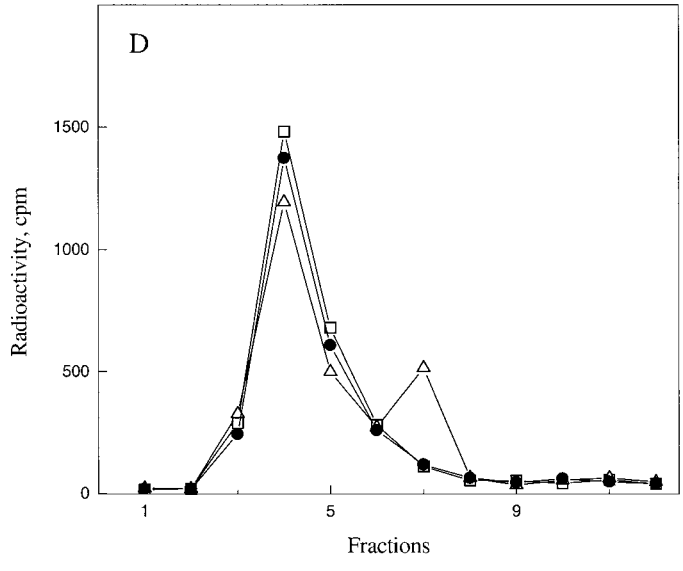
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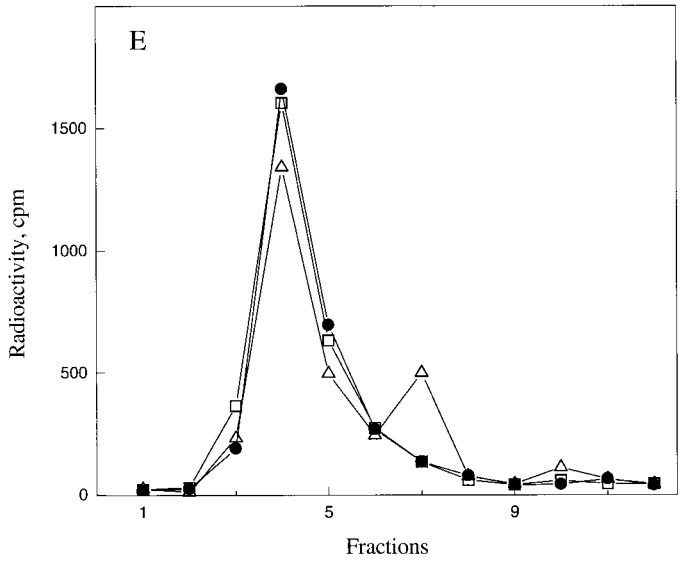
E
Radioactivity, cpm
0
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Fractions

1B). Similar results were observed with POM-SMP (not shown). Immunoblotting analysis revealed the presence of the 76-kDa subunit of complex I (34) in fractions 1–9 and 18–20 that correspond to sizes of above 700 kDa and 300–200 kDa, respectively (Fig. 1C). So the first peak contains intact complex I whereas the second peak contains a DPI-insensitive TH activity as well as a smaller form of complex I. The latter is consistent with the resolution pattern for *E. coli* complex I (35).

Stereospecificity of the transhydrogenase activities. If the SMP contain an H^+-TH activity, transfer of hydride equivalents should be between the 4A position of NAD(H) and the 4B position of NADP(H). Upon reduction of (4-^{3}H)NAD^+ a 4A-proton would be inserted forming 4B-labeled NADH. ^{3}H would be enzymatically released as ^3H_2O by 4B-specific LipDH but not by 4A-specific NR in the secondary incubations. On the other hand, in the case of transfer of 4B hydrogen from both NADPH and NADH as for the soluble TH from, e.g., *Pseudomonas,* 4A-specific NR would give radioactivity in the water peak whereas 4B-specific LipDH would not.

Transhydrogenase hydride equivalent transfer by PLM-SMP (Fig. 2A) and POM-SMP (not shown) was completely 4B-specific for NAD(H). There was no transfer of tritium to water when LipDH was used in the secondary incubations to reoxidize (4-^{3}H)NADH formed. The tritium was incorporated into water only by 4A-specific NR in the secondary incubations (Fig. 2A) and DPI in the primary incubations did not affect NADH production (Fig. 2B). Fractions 4 (Fig. 2C) and 20 (Figs. 2D and 2E) from gel filtration of solubilized PLM-SMP were also tested. We could not detect any NADPH to (4-^{3}H)NAD^+ activity in fraction 4 containing intact complex I. In fraction 20 there was only 4B-specific hydride transfer just as in the SMP (Fig. 2D) and again unaffected by DPI (Fig. 2E). In a control experiment where NADPH was omitted in the primary incubations no hydride transfer to (4-^{3}H)NAD^+ was detected (not shown). The water peak with NR seen with SMP and fraction 20 after gel filtration is therefore dependent on the presence of NADPH in the primary incubations.

DISCUSSION

The above results clearly indicate that there are two different T-D TH activities on the matrix surface of the inner membrane of mitochondria from two different plant materials. One activity is DPI-sensitive and elutes at above 700 kDa. This activity is therefore caused by complex I. It is the first time complex I is clearly shown to have a T-D activity. The second TH activity is DPI-insensitive, elutes at approx. 220 kDa after solubilization with dodecyl-maltoside and shows 4B-specificity for NADH. It is possible that this is related to the soluble non-energy-linked TH found in some bacteria (5). No activity specific for the 4A-proton of NADH, characteristic of H^+-TH, was observed in the stereospecificity assay (Fig. 2). Our results therefore indicate that the TH activity in POM-SMP previously suggested to be due to an H^+-TH (6) is most likely caused by the here described enzymes.

The two TH activities in plant mitochondria provide a non-energy-linked coupling between the reduction levels of matrix NADP and NAD. In the absence of a H^+-TH, this coupling must be quite different from that in mammalian mitochondria where the H^+-TH uses the proton-motive force to keep the NADP pool very reduced (1). Recent studies have shown that NADPH is produced or consumed in a number of important pathways in the matrix of plant mitochondria—the Krebs cycle, respiratory chain linked dehydrogenases, folate turnover, detoxification of reactive oxygen species and possibly in the regulation of enzyme activities via the thioredoxin system (7). We here report the presence of mitochondrial TH activities of a magnitude similar to those of the mitochondrial $NADP^+$-specific enzymes glutathione reductase and $NADP^+$-ICDH (36). TH activities of this magnitude might allow a rapid communication between the redox levels of the NAD and NADP pools in the matrix. Thus, in the plant cell mitochondrial oxidation of strictly NAD^+-linked substrates such as glycine or pyruvate will be able to produce NADPH for all the important NADPH-dependent processes.

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