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NAD-dependent malate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase isoenzymes play an important role in dark metabolism of various plastid types

Jan E. Backhausen[1], Susanne Vetter[1], Elisabeth Baalmann[1], Camillo Kitzmann[2], Renate Scheibe[1]

[1]Pflanzenphysiologie, Fachbereich Biologie/Chemie, Universität Osnabrück, D-49069 Osnabrück, Germany
[2]Lehrstuhl für Pflanzenphysiologie, Humboldt-Universität, D-10099 Berlin, Germany

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Abstract. Chloroplasts isolated from spinach (*Spinacia oleracea* L.) leaves and green sweet-pepper (*Capsicum annuum* L. *var. grossum* (L.) SENDT.) fruits contain NADP-dependent malate dehydrogenase (MDH; EC 1.1.1.82) and the bispecific NAD(P)-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.13). The NADP-dependent MDH and GAPDH are activated in the light, and inactive in the dark. We found that chloroplasts possess additional NAD-dependent MDH activity which is, like the NAD-dependent GAPDH activity, not influenced by light. In heterotrophic chromoplasts from red sweet-pepper fruits, the NADP-dependent MDH and the NAD(P)-GAPDH isoenzymes disappear during the developmental transition and only NAD-specific isoforms are found. Spinach chloroplasts contain both NAD/H and NADP/H at significant concentrations. Measurements of the pyridine dinucleotide redox states, performed under dark and various light conditions, indicate that NAD(H) is not involved in electron flow in the light. To analyze the contribution of NAD(H)-dependent reactions during dark metabolism, plastids from spinach leaves or green and red sweet-pepper fruits were incubated with dihydroxyacetone phosphate (DHAP). Exogenously added DHAP was oxidized into 3-phosphoglycerate by all types of plastids only in the presence of oxaloacetate, but not with nitrite or in the absence of added electron acceptors. We conclude that the NAD-dependent activity of GAPDH is essential in the dark to produce the ATP required for starch metabolism; excess electrons produced during triose-phosphate oxidation can selectively be used by NAD-MDH to form malate. Thus NADPH produced independently in the oxidative pentose-phosphate pathway will remain available for reductive processes inside the plastids.

Key words: *Capsicum* (fruit plastids) – Dark metabolism – Glyceraldehyde 3-phosphate dehydrogenase – Malate dehydrogenase – Malate valve – *Spinacia* (chloroplasts)

Introduction

During the photosynthetic light reactions, reducing equivalents are generated to drive the reductive steps in the chloroplast stroma. Ferredoxin transmits electrons either directly to the membrane-bound chloroplast enzymes (Knaff and Hirasawa 1991) or via ferrodoxin-NADP reductase (FNR) towards NADP. The generated NADPH is used by NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for the reductive step in the Calvin cycle (Leegood 1996), and by NADP-dependent malate dehydrogenase (MDH) which is a part of the malate valve (Scheibe 1987; Backhausen et al. 1994). However, chloroplasts contain NAD(H) as well, and there are several reports that both pyridine dinucleotides occur at comparable concentrations (Krause and Heber 1976; Takahama et al. 1981), but there is little knowledge about NAD-dependent chloroplast metabolism. In higher-plant chloroplasts, FNR is specific for NADP as a substrate. In vitro, its affinity for, and its V_{max} with, NAD are very low (Arnon 1969; Fredricks and Gehl 1971), and as both coenzymes compete for the active site of FNR it is unlikely that light-dependent electron flow directly towards NAD occurs in vivo.

From the slow shift between the NAD(H) and the NADP(H) pools that is often observed upon dark and light transitions it was concluded that NAD in chloroplasts acts as a reservoir for the NADP(H) pool. Indeed, chloroplasts contain NAD-kinase activity (Jalouzot et al. 1994) that is higher in the light than in the dark (Matsumura-Kadota et al. 1982). However, this is

Abbreviations: ARC = anabolic reduction charge; Chl = chlorophyll; CRC = catabolic reduction charge; DHAP = dihydroxyacetone phosphate; FNR = ferredoxin-NADP reductase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; MDH = malate dehydrogenase; OAA = oxaloacetate; 3PGA = 3-phosphoglycerate

Correspondence to: R. Scheibe;
E-mail: renate.scheibe@uni-osnabrueck.de; Fax: 49(541)9692265

probably not the only physiological function of the chloroplast NAD(H) pool, because some NAD-dependent chloroplast oxidoreductase enzymes are known. In addition to the NAD-dependent pyruvate dehydrogenase complex (Williams and Randall 1979), stromal NAD-MDH and NAD-GAPDH activities have been reported.

Strictly NAD-specific GAPDH occurs in the cytosol, while in chloroplasts the NAD- and NADP-dependent GAPDH activities are located on the same protein (Pawlizki and Latzko 1974). In contrast, the chloroplast NADP-MDH is highly specific for NADPH. With NADH as a cofactor, the purified enzyme shows only 0.2% of its NADPH-dependent activity (Scheibe and Stitt 1988). In plant cells, the presence of NAD-MDH isoforms in peroxisomes, mitochondria and in the cytosol is undoubted. Plastid preparations from various plant sources have been reported to possess an NAD-dependent MDH activity (Nainawatee et al. 1974; Amino 1992; Neuhaus et al. 1993), although one can not exclude that contaminations with other cell compartments could account for these activities.

Two dehydrogenases with overlapping coenzyme specificities that occur in the same compartment should act as a transhydrogenase and equilibrate the redox states of the NAD(H) and NADP(H) pools (Krause and Heber 1976), but in vivo this is apparently not the case. According to Takahama et al. (1981), the chloroplast NAD(H) pool remains largely oxidized in the light, and therefore probably does not function to stabilize the NADP(H) system.

The situation is different where dark metabolism is concerned. Production of NADPH in the dark occurs at the steps of glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGluDH), and NADPH is required to drive nitrite reduction (Wright et al. 1997). Thus, any NADPH consumption by MDH or GAPDH would lead to futile cycling of NADPH and to carbon loss (Siebke et al. 1991). In fact, both NADP-dependent enzymes are inactivated in the dark, when the activity of G6PDH, which controls NADPH production in the oxidative pentose-phosphate pathway (OPP), is high (Scheibe and Anderson 1981). Furthermore, the ATP demand for starch degradation can be met by NAD(H)-dependent oxidation of triose-phosphate through phosphoglycerate kinase (PGK)/GAPDH, since the concomitant removal of excess NADH can be achieved by NAD-MDH and export of malate into the cytosol.

Materials and methods

Plastid preparation and characterization. Spinach (*Spinacia oleracea* L. cv. US hybrid 424; Ferri Morse Seed Company, Modesto, Calif., USA) was grown hydroponically according to Walker (1988). Isolation of chloroplasts was as described previously (Mourioux and Douce 1981). Red and green pepper [*Capsicum annuum* L. *var. grossum* (L.) SENDT.] fruits were obtained from the local market. The plastids were isolated according to Camara (1993). The intactness of all plastid types was determined by using the latency of NADP-GAPDH. The intactness of the spinach chloroplasts was around 90%, and 78–86% for red and green pepper plastids. Contamination by other compartments was determined by marker-enzyme measurements (Quail 1979), using citrate synthase (mitochondria), UDP-glucose pyrophosphorylase (cytosol) and hydroxypyruvate reductase or catalase (peroxisomes). Chlorophyll (Chl) was determined according to MacKinney (1941). Protein was determined according to Bradford (1976), with BSA as a standard.

Experimental conditions. The plastids were kept in complete darkness before the incubation experiments were done. Isolated intact spinach chloroplasts (100 μg Chl · ml^{-1}), green pepper chloroplasts (100 μg Chl · ml^{-1}) or red pepper chromoplasts (1 mg protein · ml^{-1}) were incubated at room temperature (22 °C) in a mixture of 330 mM betaine, 50 mM Hepes-KOH (pH 7.6), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.2 mM KH_2PO_4 and further components as indicated.

Gel-filtration experiments. Plastid samples were diluted fourfold in a medium consisting of 4 mM EDTA, Triton X-100 (0.05%, v/v) adjusted to pH 8.0 with KOH. The samples were mixed and centrifuged for 5 min at 10 000 *g*. The supernatant (2 ml) was passed through a 0.2-μm filter and immediately subjected to gel filtration, using a fast protein liquid chromatography (FPLC) system (Pharmacia, Freiburg, Germany) and a Superdex-200 column (Pharmacia) with a flow rate of 1.5 ml · min^{-1} at 8 °C. Elution was achieved with a buffer consisting of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 150 mM KCl. The fractions were collected and assayed immediately.

Enzyme measurements. For the determination of the activation states of the redox-modulated enzymes, the extraction method described for NADP-MDH in Backhausen et al. (1994) was used. Full activation and activity assay of NADP-MDH (Backhausen et al. 1994) and GAPDH (Baalmann et al. 1994) were done as described. The NAD- and NADP-dependent GAPDH activities were determined in the same assay medium, with either NADH or NADPH as coenzyme. The NAD-MDH was measured as in Scheibe and Stitt (1988).

Measurements of metabolites. Samples for the measurements of malate and 3-phosphoglycerate (3PGA) were obtained at the indicated time by adding 400 μl of the incubation medium into 40 μl of 65% (v/v) perchloric acid, 0.1% (v/v) Triton X-100, containing 4 mM EDTA. After 20 min at room temperature, the samples were frozen in liquid nitrogen. Prior to metabolite measurements the samples were adjusted to pH 7.8 with a mixture of 0.2 M triethanolamine, 2 M $KHCO_3$, and 2 M K_2CO_3 (pH 9.0). 3-phosphoglycerate (3PGA; Stitt et al. 1989) and malate (Backhausen et al. 1994) were determined enzymatically. The samples for the determination of pyridine dinucleotides were obtained as described in Backhausen et al. (1994), using silicone-oil centrifugation in order to concentrate the samples. Six samples were pooled for each determination. The determinations of NAD and NADP, and of NADH and NADPH were performed by a cyclic assay according to Slater and Sawyer (1962). From the measured concentrations of NAD(H) and NADP(H), the anabolic reduction charge (ARC) and the catabolic reduction charge (CRC) were calculated according to Andersen and Von Meyenburg (1977): ARC = NADPH/(NADP + NADPH) and CRC = NADH/(NAD + NADH).

Results

Light-dependent changes in enzyme-activation states and in the pool sizes of NAD(H) and NADP(H) in isolated spinach chloroplasts. Isolated intact spinach chloroplasts were used to estimate the dark and light activities of MDH and GAPDH, and the changes in the redox states

Table 1. Enzyme activities and redox states of the NAD(P)/H pool in isolated intact spinach chloroplasts. Enzyme activities are given as μmol · (mg Chl)$^{-1}$ · h^{-1}. The redox states of the pyridine-dinucleotide pools are given as ARC = NADPH/(NADPH + NADP) and CRC = NADH/(NADH + NAD). All light samples were taken after 7 min of illumination as described in *Materials and methods*. The values are means (± SD) from three to seven independent chloroplast preparations. *n.d.*, not determined

	Dark	Light (control)	Light +5 μM antimycin A	Light +2 mM nitrite
NADP-MDH	2 ± 0.8	95 ± 10	150 ± 34	11 ± 3
NAD-MDH	80 ± 15	80 ± 17	n.d.	n.d.
NADP-GAPDH[a]	210 ± 20	650 ± 59	n.d.	625
NAD-GAPDH[a]	300 ± 22	300 ± 30	n.d.	n.d.
ARC	0.34 ± 0.06	0.45 ± 0.05	0.54 ± 0.01	0.38 ± 0.03
CRC	0.28 ± 0.04	0.30 ± 0.04	0.31 ± 0.06	0.31 ± 0.04

[a]At 55 μM 1,3bisPGA and 320 μM NAD(P)H

of the pyridine-dinucleotide pools. The NADP-MDH was essentially inactive in the dark, while the NAD-MDH activity was not influenced by dark-light transitions (Table 1). For both NAD- and NADP-GAPDH, when assayed under standard conditions, significant activities were found in darkened chloroplasts. The NAD-GAPDH activity remained unchanged upon transition from dark to light, and only the NADP-dependent activity was increased threefold in the light (Table 1).

Both NAD(H) and NADP(H) were present in significant amounts in the chloroplast stroma. The concentration of NAD + NADH was 3.7 ± 0.7 nmol · (mg Chl)$^{-1}$, NADP + NADPH was 7.2 ± 1.5 nmol · (mg Chl)$^{-1}$, and their total concentrations were not significantly (less than 10%) altered during the time of the experiments. In the dark, 34% of the NADP(H) pool was reduced (ARC = 0.34). Its redox state increased significantly up to 0.45 upon illumination (Table 1). The CRC was more oxidized, and did not change upon illumination (Table 1). The coupling between the two pools was analyzed by either applying extreme electron drainage (2 mM nitrite) or massive overreduction (antimycin A) to the plastids (Backhausen et al. 1994; Holtgrefe et al. 1997). For the ARC, significant deviations from the steady-state values were observed, as was also reflected by the activation state of NADP-MDH, while, in contrast, the CRC was rather stable (Table 1).

Occurrence of NAD- and NADP-dependent enzyme activities in the various plastid types. The method for chloroplast isolation used here yields extremely pure plastids. Contamination with cytosol, vacuoles or mitochondria was below the limits of detection, and contamination with peroxisomes was less than 1% as assayed with hydroxypyruvate reductase or catalase as marker enzymes. In order to check the localization of the NAD-MDH, its latency properties were compared with those of NADP-GAPDH (as stromal marker enzyme; Table 2). About 85% of the NADP-GAPDH activity was latent, reflecting the intactness of the plastid preparation used. With NAD-MDH, 60% of the activity was latent, indicating its main localization in the stroma. Of the non-latent activity 15% must be attributed to non-intact chloroplasts (as compared to the NADP-GAPDH distribution). The remaining activity (about 25% of the NAD-MDH) must be due to contaminating peroxisomes, since peroxisomal enzymes are reported to show no latency under these conditions (Heupel and Heldt 1994).

Table 2. Latency of NAD-MDH and NADP-GAPDH activities. Isolated intact spinach chloroplasts were assayed either in their standard assay medium (*shocked*) or in the same medium with 330 mM betaine (*intact*). The activities are given as μmol · (mg Chl)$^{-1}$ · h^{-1}

	Intact	Osmotically shocked
NADP-GAPDH	31 ± 6	190 ± 28
NAD-MDH	35 ± 12	90 ± 19

To further characterize the NAD- and NADP-dependent plastid enzymes in samples from different sources, gel-filtration experiments were performed. Ruptured samples of dark-adapted chloroplasts from spinach leaves, green and red pepper fruits were applied to a Superdex-200 column, and the fractions were assayed for NAD- and NADP-dependent MDH and GAPDH activities (Fig. 1). Both GAPDH activities and that of NAD-MDH were assayed directly in the fractions without any preincubation. Since NADP-MDH is essentially inactive under these conditions, its activity was assayed after incubation with reduced DTT.

The elution profile obtained with spinach chloroplasts was characterized by a single peak of both, NAD- and NADP-dependent GAPDH activities at 600 kDa, and the same pattern was obtained with green pepper fruit plastids (Fig. 1A,B). In red pepper fruit plastids only the minor peak of GAPDH activity at 600 kDa exhibited an NADP- to NAD-dependent activity ratio similar to that found in plastids from green peppers and spinach, while most of the NAD-GAPDH activity eluted at 150 kDa (Fig. 1C). In contrast to the 150-kDa peak of light-activated chloroplast GAPDH, this activity was exclusively NAD-dependent, indicating that a new isoenzyme appears during the developmental transition from green to red pepper fruits.

The NADP-MDH activities were found to elute exclusively around 80 kDa with all three plastid types, but NAD-MDH activity often appeared as two peaks: most of the activity co-eluted with the NADP-MDH and some activity was also found in the exclusion volume, corresponding to a molecular mass of more than

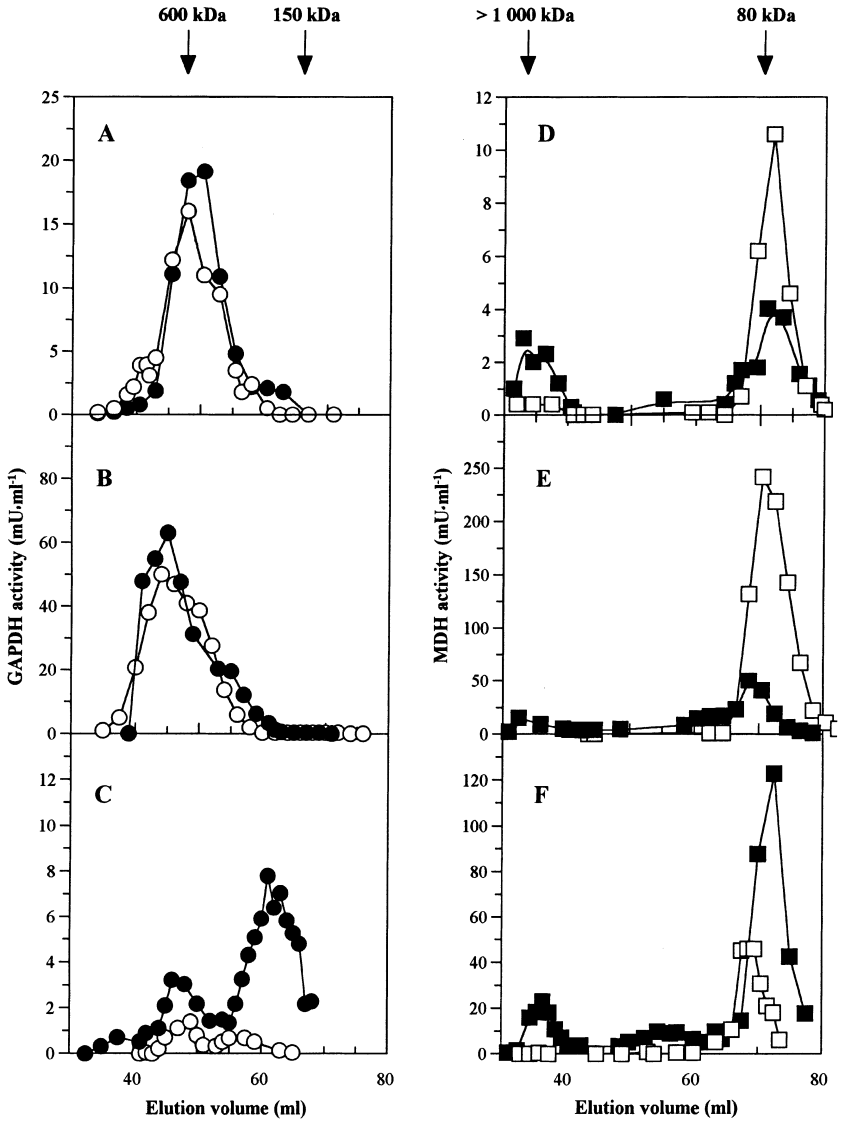

Fig. 1A–F. Gel filtration of isolated plastids from red or green pepper fruits and spinach leaves. Plastids were isolated from spinach leaves (**A, D**), green pepper (**B, E**) and red pepper fruits (**C, F**), respectively. Darkened samples from the plastid preparations were applied to the Superdex-200 column. Eluted fractions were collected and assayed immediately for NAD-GAPDH (●), NADP-GAPDH (○) and NAD-MDH (■) activities. In order to assay NADP-MDH (□) activity, the samples were activated with DTT_{red}, as described in *Materials and methods*

1000 kDa. This latter peak was probably due to peroxisomal contamination, since peroxisomal proteins remain associated even in the presence of low amounts of detergent (Heupel et al. 1991), as used for rupturing the plastid preparations. For spinach chloroplasts, the portion of NAD-dependent activity in the exclusion volume corresponds to the 25% of peroxisomal contamination as estimated in the latency experiments (Table 2).

Electron fluxes during dihydroxyacetone phosphate (DHAP) oxidation in the dark. In order to determine the contributions of NAD-dependent MDH and GAPDH activities in the dark metabolism, the oxidation of exogenously added DHAP was studied in plastids from the various sources. As shown in Fig. 2, incubation of spinach chloroplasts with DHAP alone did not lead to any 3PGA formation, indicating the necessity to remove the generated reducing equivalents. As electron acceptors, oxaloacetate (OAA) and nitrite were used. Since the NADP-dependent MDH is inactive in the dark, OAA reduction should be catalyzed by the stromal

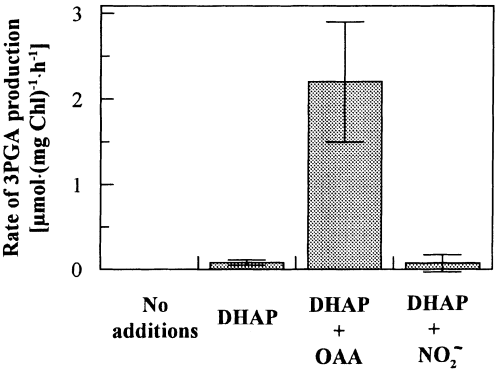

Fig. 2. Rates of 3PGA production in isolated intact spinach chloroplasts in the dark. The chloroplasts were incubated without further additions, or with the indicated additions of 0.5 mM DHAP alone or together with either 0.2 mM OAA or 0.2 mM nitrite

NAD-MDH. In contrast, for nitrite reduction reduced ferredoxin is required which can exclusively be formed from NADPH by reversed electron flow via FNR (Bowsher et al. 1993). Only when the electrons were

removed by the addition of OAA, was 3PGA formed at a rate of 2 μmol · (mg Chl)$^{-1}$ · h^{-1} (Fig. 2), while incubating the plastids with DHAP plus nitrite did not lead to 3PGA formation (Fig. 2). During the OAA-dependent oxidation of DHAP malate was formed at rates which were stable for at least 15 min. For OAA, a concentration of 50 μM was saturating, for DHAP the reaction was saturated at 100–200 μM (data not shown). With osmotically shocked plastids 3PGA was not formed under any of these conditions (data not shown).

The concentration dependence of malate and 3PGA production was compared in the various types of plastids. In spite of the differences in enzyme equipment, all three types of plastid were able to catalyze DHAP- and OAA-dependent electron flow (Fig. 3). The expected 1:1 ratio of 3PGA and malate production, however, was only obtained with red peppers (Fig. 3C). In preparations of green plastids, especially in those from pepper fruits, less 3PGA than malate was accumulated (Fig. 3B), indicating that some further metabolism of 3PGA, probably for amino- or fatty-acid synthesis takes place.

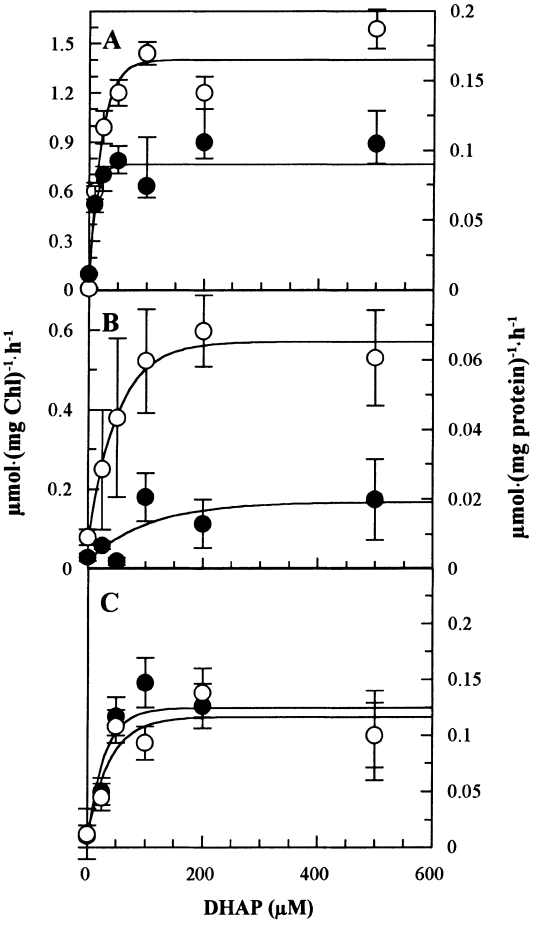

Fig. 3A–C. Rates of DHAP- and OAA-dependent formation of 3PGA and malate by the various plastid types. Isolated chloroplasts from spinach leaves (**A**), green (**B**) or red (**C**) pepper fruits were incubated with 50 μM OAA plus DHAP at the indicated concentrations. Samples for the determinations of 3PGA (●) and malate (○) were taken every 5 min. The rates were calculated from the linear part of the curves

Discussion

In this work, we intended to obtain information about NAD-dependent plastid metabolism, focussing on three topics: (i) the stromal localization of NAD-dependent enzyme activities, (ii) changes in the redox states of the NAD/H or NADP/H pools and (iii) the physiological conditions for NAD-dependent electron fluxes.

Capacities of the NAD- and NADP-dependent dehydrogenases in isolated chloroplasts. As is known, the chloroplast GAPDH can use both NAD(H) and NADP(H) (Schulman and Gibbs 1968) with NAD being the preferred coenzyme for the 600-kDa dark form. The occurrence of NAD-MDH in chloroplasts has often been questioned (Siebke et al. 1991), because in earlier studies neither loss of stromal protein nor contamination with NAD-MDH from other compartments (Nainawatee et al. 1974; Amino 1992) could be completely ruled out. The chloroplasts used for our experiments were extremely pure. Latency experiments clearly demonstrate that about two-thirds of the NAD-dependent MDH is localized in the stroma and only 25% of the NAD-MDH must be attributed to peroxisomal contamination. In contrast to the soluble NAD- and the NADP-MDH that eluted around 80 kDa, the peroxisomal contamination eluted as a high-molecular-mass complex (Fig. 1A). The total stromal NAD-dependent MDH activity in the spinach chloroplast was around 100 μmol · (mg Chl)$^{-1}$ · h^{-1}, which is slightly lower than the NADP-MDH capacity. However, it accounts for only a few percent of the total cellular NAD-MDH activity which is usually about 4000 μmol · (mg Chl)$^{-1}$ · h^{-1} (Scheibe and Stitt 1988).

For chloroplasts from green pepper fruits the elution pattern is similar, only the ratio of NADP- to NAD-MDH is higher (Fig. 1). Since the capacity of fruit chloroplasts to fix CO_2 is lower than in leaf plastids (Piechulla et al. 1987), the increased NADP-MDH activity is consistent with the function of the malate valve to remove excess electrons in a situation of overreduction (Backhausen et al. 1994) as caused by the limited electron usage for CO_2 assimilation.

In contrast, chromoplasts are strictly heterotrophic and both NADP-dependent enzyme activities have largely disappeared. In the case of MDH, the expression of the NADP-dependent isoenzyme has decreased and the NAD-dependent activity becomes predominant. In the case of GAPDH, most of the NADP- and NAD-dependent activities at 600 kDa have disappeared, and a new, exclusively NAD-dependent GAPDH appeared at 150 kDa (Fig. 1C). The gene for such an NAD-dependent plastidic GAPDH isoform has been recently found by sequencing gymnosperm cDNA (Meyer-Gauen et al. 1994).

Is NAD(H) preferentially required for dark or for light metabolism? In the stroma of spinach chloroplasts, both pyridine dinucleotides [NAD(H) and NADP(H)] are found at significant concentrations, but with large differences in their redox states. The CRC is more

oxidized both under dark and light conditions, and is only slightly increased upon illumination. Furthermore, when extreme redox situations are applied to the spinach chloroplasts, only the ARC is affected, while the CRC does not change. This difference between the CRC and the ARC underlines the absence of coupling between the two pools, and shows that the simultaneous presence of NAD- and NADP-MDH and GAPDH activities does not result in any significant transhydrogenase activity in the light. However, it must be considered that the rate of electron flow via the NADP(H) pool during photosynthesis in the light lies in the range of 100 μmol · (mg Chl)$^{-1}$ · h^{-1}, while the rates of DHAP oxidation in the dark reached their maximum at about 2 μmol · (mg Chl)$^{-1}$ · h^{-1} (Fig. 3).

This raises the question of why there is no major flux through the NAD(H) pool during photosynthesis, although the NAD-dependent enzymes are not inactivated in the light (Table 1). The estimation of enzyme activities in vitro is generally done under optimized conditions, in order to obtain activities that are close to the V_{max} of the respective enzyme. It is very unlikely that in vivo the enzymes encounter similar conditions, and this causes large discrepancies between the in-vitro enzyme activity and the substrate flux in vivo. In particular, the reaction of MDH is, apart from the redox-regulation of the NADP-isoenzyme, strongly influenced by the in-vivo ratio between substrates and products, and the more-oxidized CRC will restrict the formation of NAD in the light. Further, the substrate concentrations have a large impact, since they are normally not saturating. This especially decreases the rate of glyceraldehyde 3-phosphate (GAP) formation as catalyzed by GAPDH. For example, NADP-GAPDH when assayed under V_{max} conditions (Table 1) shows a significant dark activity, but due to the high K_m value (K_m[1,3bisPGA] = 100 μM) of the oxidized 600-kDa form and the low dark concentration of 1,3bisPGA (0.05 μM; Baalmann et al. 1995) the carbon flux involving NADPH consumption in vivo must be close to zero.

Physiological significance of NAD-dependent metabolism in the dark. Several lines of evidence indicate that dark conditions clearly promote NAD-dependent MDH and GAPDH reactions. In autotrophic plastids such as those from spinach leaves, only the NADP-dependent MDH and GAPDH are subjected to redox modulation. In the classical sense, this means that they are activated by light, but in vivo, the fact that they are inactivated in the dark may be more important. Non-green plastids possess negligible activities of NADP-MDH and NADP-GAPDH (Neuhaus et al. 1993). For the red-pepper fruit plastids used here we found that, in the case of MDH, this appears to be the result of a shift in the isoenzyme-expression ratio favouring the NAD-dependent one, while in the case of GAPDH, a new isoenzyme is expressed.

Apart from the large differences in their NADP-dependent enzymes, intact plastids from all three sources were able to oxidize DHAP into 3PGA at significant rates, but only when OAA, and not when nitrite was added. Under dark conditions, these electron acceptors should be very specific for either NADH or NADPH. Since the NADP-MDH is inactive in the dark, OAA reduction is only possible with NADH, while nitrite reduction requires reduced ferredoxin which is exclusively formed from NADPH supplied by G6PDH and 6PGluDH via FNR (Bowsher et al. 1993; Wright et al. 1997). Similar differences between the effects of nitrite and OAA were observed by Singh et al. (1993) during their analysis of dark CO_2 production from exogenously added glucose. In fact, only the use of NAD as coenzyme for MDH and GAPDH during OAA-dependent DHAP oxidation allows for uncoupled production of NADH, NADPH (via glucose-6-phosphate dehydrogenase) and ATP (Fig. 4) as required for several anabolic reactions in dark metabolism such as nitrite assimilation (Robinson 1988; Bowsher et al. 1993), de-novo synthesis of fatty acids (Kleppinger-Sparace et al. 1992), starch biosynthesis in heterotrophic plastids (Neuhaus et al. 1993) or carbohydrate degradation via phosphofructokinase in autotrophic plastids (Stitt and Heldt 1981). Thus, NAD-MDH drives a dark form of the malate valve, balancing the redox state of the NAD(H) pool and linking this to electron transfer into the cytosol (Fig. 4).

Further experimental evidence for the onset of these reactions during starch degradation comes from the

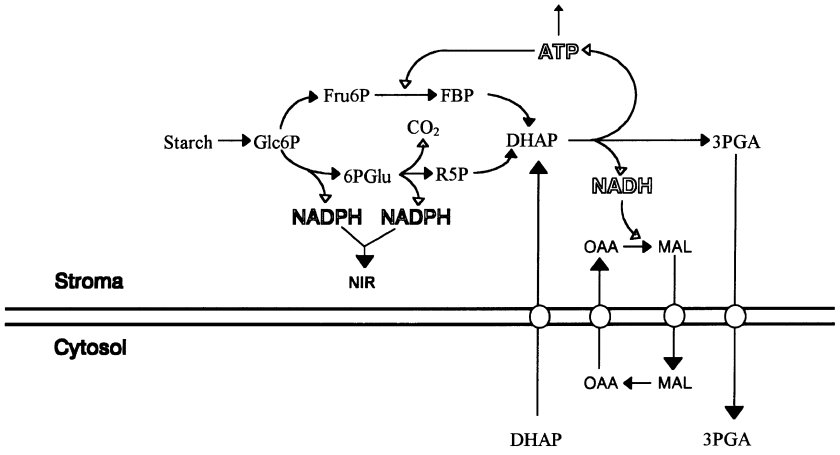

Fig. 4. Scheme summarizing the suggested roles of the NAD-dependent MDH and GAPDH activities in darkened plastids. During starch degradation, NADPH is produced by the oxidative pentose-phosphate pathway (OPP). The NAD-GAPDH and phosphoglycerate kinase oxidize exogenous DHAP or the triose-P formed in the OPP, and produce NADH and ATP in a coupled reaction. The NADH that is not required for other chloroplast reactions is consumed by NAD-MDH, the malate in turn can be exported from the chloroplast. *FBP*, fructose-1,6-bisphosphate; *MAL*, malate; *NIR*, nitrite reductase; *6PGlu*, 6-phosphogluconate; *R5P*, ribulose 5-phosphate

work of Neuhaus and Schulte (1996). In chloroplasts from *Mesembryanthemum crystallinum*, starch degradation was promoted by the addition of OAA, which caused a strong decrease in the DHAP/3PGA ratio. The stimulatory effect of OAA on starch degradation was comparable to that achieved by the addition of ATP. It is true that import of ATP via an ATP/ADP translocator is possible (Neuhaus et al. 1997), but unlike the situation in heterotrophic plastids where uniport of Pi was found (Neuhaus and Maaß 1996), in chloroplasts this will result in increased stromal Pi levels which would cause serious delays in the onset of CO_2 assimilation.

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References

Amino S (1992) Intracellular conversion of malate and localization of enzymes involved in the metabolism of malate in photoautotrophic cell cultures of *Chenopodium rubrum*. Z Naturforsch 47c: 545–552

Andersen KB, Von Meyenburg K (1977) Changes of nicotinamide adenine nucleotides and adenylate energy charge as regulatory parameters of the metabolism in *Escherichia coli*. J Biol Chem 252: 4151–4156

Arnon D (1969) Role of ferredoxin in photosynthesis. Naturwissenschaften 56: 295–305

Baalmann E, Backhausen JE, Kitzmann C, Scheibe R (1994) Regulation of NADP-dependent glyceraldehyde 3-phosphate dehydrogenase activity in spinach chloroplasts. Bot Acta 107: 313–320

Baalmann E, Backhausen JE, Rak C, Vetter S, Scheibe R (1995) Reductive modification and nonreductive activation of purified spinach chloroplast NADP-dependent glyceraldehyde-3-phosphate dehydrogenase. Arch Biochem Biophys 324: 201–208

Backhausen JE, Kitzmann C, Scheibe R (1994) Competition between electron acceptors in photosynthesis: Regulation of the malate valve during CO_2 fixation and nitrite reduction. Photosynth Res 42: 75–86

Bowsher CG, Hucklesby DP, Emes MJ (1993) Induction of ferredoxin-$NADP^+$ oxidoreductase and ferredoxin synthesis in pea root plastids during nitrate assimilation. Plant J 3: 463–467

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 74: 248–254

Camara B (1993) Plant phytoene synthase complex: component enzymes, immunology, and biogenesis. Methods Enzymol 214: 352–365

Fredricks WW, Gehl JM (1971) Stimulation of the transhydrogenase activity of spinach ferredoxin-nicotinamide adenine dinucleotide phosphate reductase by ferredoxin. J Biol Chem 246: 1201–1205

Heupel R, Heldt HW (1994) Protein organization in the matrix of leaf peroxisomes. A multi-enzyme complex involved in photorespiratory metabolism. Eur J Biochem 220: 165–172

Heupel R, Markgraf T, Robinson DG, Heldt HW (1991) Compartmentation studies on intact spinach leaf peroxisomes. Evidence for channeling of photorespiratory metabolites in peroxisomes devoid of intact boundary membranes. Plant Physiol 96: 971–979

Holtgrefe S, Backhausen JE, Kitzmann C, Scheibe R (1997) Regulation of steady-state photosynthesis in isolated intact chloroplasts under constant light: responses of carbon fluxes, metabolite pools and enzyme-activation states to changes of the electron pressure. Plant Cell Physiol 38: 1207–1216

Jalouzot R, Pou MA, Aubry C, Laval-Martin D (1994) The NAD kinase: a phosphoryltransferase displaying an oxido-reductase activity. An electrophoretic study. Arch Biochem Biophys 309: 281–287

Kleppinger-Sparace KF, Stahl RJ, Sparace SA (1992) Energy requirements for fatty acid and glycerolipid biosynthesis from acetate by isolated pea root plastids. Plant Physiol 98: 723–727

Knaff DB, Hirasawa M (1991) Ferredoxin-dependent chloroplast enzymes. Biochim Biophys Acta 1056: 93–125

Krause GH, Heber U (1976) Energetics of intact chloroplasts. In: Barber J (ed) The intact chloroplast. Elsevier, Amsterdam, pp 171–214

Leegood RC (1996) Primary photosynthate production: physiology and metabolism. In: Zamski E, Schaffer AA (eds) Photoassimilate distribution in plants and crops. Source-sink relationships. Dekker, New York, pp 21–41

MacKinney G (1941) Absorption of light by chlorophyll solutions. J Biol Chem 140: 315–322

Matsumura-Kadota H, Muto S, Miyachi S (1982) Light-induced conversion of NAD to NADP in *Chlorella* cells. Biochim Biophys Acta 679: 300–307

Meyer-Gauen G, Cerff R, Schnarrenberger C, Martin W (1994) Molecular characterization of a novel, nuclear-encoded, NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase in plastids of the gymnosperm *Pinus sylvestris* L. Plant Mol Biol 26: 1155–1166

Mourioux G, Douce R (1981) Slow passive diffusion of orthophosphate between intact isolated chloroplasts and the suspending medium. Plant Physiol 76: 470–473

Nainawatee HS, Mehta SL, Das NB (1974) Soluble and chloroplast malate dehydrogenase isoenzymes of *Triticum aestivum*. Phytochemistry 13: 553–557

Neuhaus HE, Maaß U (1996) Unidirectional transport of orthophosphate across the envelope of isolated cauliflower-bud amyloplasts. Planta 198: 542–548

Neuhaus HE, Schulte N (1996) Starch degradation in chloroplasts isolated from C3 or CAM (crassulacean acid metabolism)-induced *Mesembryanthemum crystallinum* L. Biochem J 318: 945–953

Neuhaus HE, Batz O, Thom E, Scheibe R (1993) Purification of highly intact plastids from various heterotrophic plant tissues: analysis of enzymic equipment and precursor dependency for starch biosynthesis. Biochem J 296: 395–401

Neuhaus HE, Thom E, Möhlmann T, Steup M, Kampfenkel K (1997) Characterization of a novel eukaryotic ATP/ADP translocator located in the plastid envelope of *Arabidopsis thaliana* L. Plant J 11: 73–82

Pawlizki K, Latzko E (1974) Partial separation and interconversion of NADH- and NADPH-linked activities of purified glyceraldehyde 3-phosphate dehydrogenase from spinach chloroplasts. FEBS Lett 42: 285–288

Piechulla B, Glick RE, Bahl H, Melis A, Gruissem W (1987) Changes in photosynthetic capacity and photosynthetic protein pattern during tomato fruit ripening. Plant Physiol 84: 911–917

Quail PH (1979) Plant cell fractionation. Annu Rev Plant Physiol 30: 425–484

Robinson JM (1988) Spinach leaf chloroplast CO_2 and NO_2^- photoassimilations do not compete for photogenerated reductant. Plant Physiol 88: 1373–1380

Scheibe R (1987) $NADP^+$-malate dehydrogenase in C3-plants: regulation and role of a light-activated enzyme. Physiol Plant 71: 393–400

Scheibe R, Anderson LE (1981) Dark modulation of NADP-dependent malate dehydrogenase and glucose-6-phosphate dehydrogenase in the chloroplast. Biochim Biophys Acta 636: 58–64

Scheibe R, Stitt M (1988) Comparison of NADP-malate dehydrogenase activation, Q_A reduction, and O_2 evolution in spinach leaves. Plant Physiol Biochem 26: 473–481

Schulman MD, Gibbs M (1968) D-Glyceraldehyde 3-phosphate dehydrogenases of higher plants. Plant Physiol 43: 1805–1812

Siebke K, Laisk A, Neimanis S, Heber U (1991) Regulation of chloroplast metabolism in leaves: evidence that NADP-dependent glyceraldehyde phosphate dehydrogenase but not ferredoxin-NADP reductase controls electron flow to phosphoglycerate in the dark-light transition. Planta 185: 337–343

Singh KK, Chen C, Gibbs M (1993) Photoregulation of fructose and glucose respiration in the intact chloroplasts of *Chlamydomonas reinhardii* F-60 and spinach. Plant Physiol 101: 1289–1294

Slater T, Sawyer B (1962) A colorimetric method for estimating the pyridine nucleotide content of small amounts of animal tissue. Nature 193: 454–456

Stitt M, Heldt HW (1981) Physiological rates of starch breakdown in isolated intact spinach chloroplasts. Plant Physiol 68: 755–761

Stitt M, Lilley M, Gerhardt R, Heldt HW (1989) Metabolite levels in specific cells and subcellular compartments of plant leaves. Methods Enzymol 174: 518–554

Takahama U, Shimizu-Takahama M, Heber U (1981) The redox state of the NADP system in illuminated chloroplasts. Biochim Biophys Acta 637: 530–539

Walker DA (1988) The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis. 2nd edn, Oxygraphics Limited, Sheffield

Williams M, Randall DD (1979) Pyruvate dehydrogenase complex from chloroplasts of *Pisum sativum* L. Plant Physiol 64: 1099–1103

Wright DP, Huppe HC, Turpin DH (1997) *In vivo* and *in vitro* studies of glucose-6-phosphate dehydrogenase from barley root plastids in relation to reductant supply for NO_2-assimilation. Plant Physiol 114: 1413–1419