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Modulation of the glyceraldehyde-3-phosphate dehydrogenase isozyme activities in spinach leaves

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Abstract

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NAD- and NADP-dependent glyceraldehyde-3-phosphate dehydrogenase isozymes (EC 1.2.1.12 and EC 1.2.1.13, respectively) from spinach leaves were separated by inverse ammonium sulphate gradient solubilization. Manipulation of the isozyme activities by addition of NAD^+ or $NADP^+$ at the moment of extraction was compared to *in vivo* treatments (DCMU, etiolated cotyledons). The DCMU effect showed that only one chloroplast isozyme is activated by photosynthesis-derived changes in nucleotide levels. Addition of NAD^+ during extraction of plants sampled in the light phase resulted in chloroplast isozyme activity ratios similar to those observed with extracts from dark control plants, whereas addition of $NADP^+$ to extracts from plants sampled in darkness resulted in activity ratios lower than those from the light control extracts. Modulation of the NAD^+-specific cytosolic isozyme by NAD^+ was confirmed by the isozyme activities obtained from etiolated cotyledons. Manipulation of the $NAD^+/NADP^+$ ratio at the moment of extraction thus reflected differences correlated with potential *in vivo* modulation of the isozymes by nucleotide ratios.

Key words: Spinacia oleracea L., glyceraldehyde-3-phosphate dehydrogenase isozymes. NAD^+, $NADP^+$, DCMU.

Introduction

The higher plant glyceraldehyde-3-phosphate dehydrogenase (GPD) isozyme set is composed of a NAD^+-specific cytosolic (glycolysis-linked) enzyme (EC 1.2.1.12) and two chloroplast isozymes (EC 1.2.1.13), each one accepting both NAD(H) and NADP(H) as cofactors (Cerff and Chambers 1978; Cerff 1979; de Looze and Wagner 1983 a, b; Bonzon et al. 1987). Using the inverse ammonium sulphate gradient solubilization technique, Wagner (1976) and de Looze and Wagner (1983 b) demonstrated that the isozyme activities from crude extracts of *Chenopodium rubum* leaves showed diurnal variations which could be chemically modulated at the moment of extraction. Changes

in the isozyme activities were also observed during the course of photoperiodic floral induction and the acclimation processes in spinach leaves (Bonzon et al. 1987).

It was suggested that the observed changes in GPD isozyme activities were correlated with either diurnal rhythms in the redox state (Wagner and Frosh 1974; Bonzon et al. 1983; Simon et al. 1982, 1984; de Looze and Wagner 1983 b) or in energy charge (Wagner et al. 1974; Bonzon et al. 1981; Hampp et al. 1982; Lavergne and Champigny 1985) or a modification of the chloroplast ATP/NADPH ratio (Bonzon et al. 1987; see Melis et al. 1985). These interpretations assumed that the isozyme activities obtained from crude homogenates, in which microenvironments, compartments and effector concentrations had been radically altered, reflected the actual state of activation of each isozyme in its *in vivo* situation. However, the modulation of the GPD isozyme activities is dependent on a complex regulator feedback loop. While participating in the dynamic equilibrium determining the pyridine nucleotide ratios in the various cellular compartments, the isozymes themselves are in turn regulated by pyridine nucleotide ratios (Cerff and Chambers 1978). In the chloroplast compartment, an additional control depends on the ferredoxin-thioredoxin light enzyme activating system (Wolosiuk and Buchanan 1977; see also Buchanan 1980 and Jacquot 1984).

In this context, it was considered to be of interest to examine to what extent the GPD isozyme activities are influenced by a manipulation of the $NAD^+/NADP^+$ ratio at the moment of extraction and to compare these effects not only to the control extracts, but also to those of *in vivo* treatments having similar effects, such as inhibition of photosynthesis by DCMU or etiolation.

Material and methods

Plant material, growth conditions and treatments

Spinacia oleracea L. cv. Nobel (spinach) plants were cultivated on soil, four plants to a pot, in growth chambers under standard conditions of illumination (20 Wm^{-2} provided by Sylvania fluorescent lamps "daylight", 40 W), temperature (21 °C), and humidity (70% relative humidity during the day and 50% during the night). Four-week old vegetative plants kept in a 8 h light: 16 h dark cycle were used. Etiolated seedlings were harvested 10 days after germination on vermiculite in the dark at 21 °C. Modulation of the isozyme activities was studied in extracts made with extraction buffer containing 0.2 mM of either NAD^+ or $NADP^+$ in addition to the other components (de Looze and Wagner 1983 b). The effect of DCMU was tested by spraying each plant once with 2 ml of a 0.1 mM DCMU solution at 8.00 h, 16.00 h (end of the night and light period, respectively) and again at 8.00 h the next day. Extraction was carried out at 16.00 h, i.e. 8 h after the last treatment.

Extraction procedure, inverse ammonium sulphate gradient solubilization, and enzyme assay

These procedures were carried out according to de Looze and Wagner (1983 a, b), as modified by Bonzon et al. (1987). The crude extracts were obtained from about 3–4 g fresh weight of primary leaves or 2 g of etiolated cotyledons (excised under green safe light). Total activities in the fractions after inverse ammonium sulphate gradient solubilization were expressed as µmol NAD(P)H oxidized min^{-1}.

Peak area measurement

Total NAD^+- and $NADP^+$-dependent GPD I activities (see Fig. 1) were estimated as the area under their respective elution profiles up to the last fraction at 60% ammonium sulphate saturation (de Looze and Wagner 1983 b). Remaining activities are NAD^+- and $NADP^+$-GPD II, respectively.

The contribution of the NAD^+-specific cytosolic enzyme (NAD^+-GPD cyt.) to the total NAD^+-GPD I activity was calculated according to Bonzon et al. (1987). Peak area measurements were made either by planimetry or using a computer-aided method.

Statistics

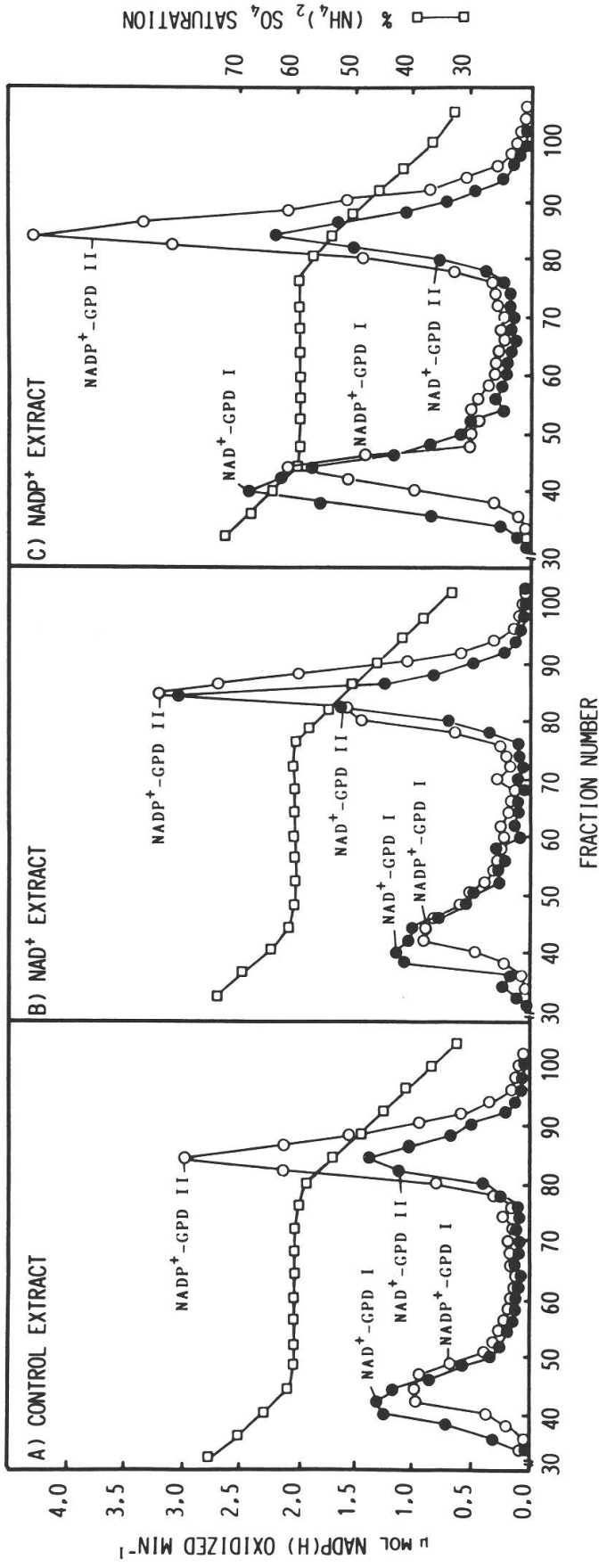
Each experiment was repeated at least three times with different batches of plants. Recoveries after ammonium sulphate gradient solubilization ranged between 85 and 105% of the activity in the crude extract for both NAD^+- and $NADP^+$-dependent GPD activities. The pattern of the isozyme activities was very stable from experiment to experiment. Data shown are those from one representative set of experiments. Standard errors for the area under the activity peaks (see Results) were calculated from a minimum of four independent experiments.

Results

The isozyme activities for three different crude extracts from 4-week old primary leaves sampled at the 7th h of darkness ("dark extracts") are shown in Fig. 1. In each case, the $NADP^+$-GPD I and the two GPD II isozymes are of chloroplast origin, whereas the NAD^+-GPD I activity peak is a mixture of the NAD^+-specific (glycolysis-linked) enzyme and the NAD^+-GPD I chloroplast isozyme (Bonzon et al. 1987). As compared to the control extract (Fig. 1 A), the NAD^+ extract (Fig. 1 B) showed an increased NAD^+-GPD II activity, whereas the $NADP^+$ extract (Fig. 1 C) resulted in increased activities of all isozymes. A similar experiment with plants sampled at the 6th h of light ("light extracts") resulted, as compared to the corresponding control extract, in increased activities of the NAD^+- and $NADP^+$-GPD I isozymes for the NAD^+ ex-

Table 1. Effect of NAD^+, $NADP^+$ and DCMU on the % $NADP^+$-GPD I/$NADP^+$-GPD II chloroplast activity ratio and on the % NAD^+-GPD cyt./NAD^+-GPD I+II cytosolic activity ratio from spinach primary leaves and comparison with etiolated cotyledons. Ratios calculated from the total activities measured as the area under the respective elution profile as described in Material and methods.

Conditions		$\frac{NADP^+\text{-GPD I}}{NADP^+\text{-GPD II}}$ (%)	$\frac{NAD^+\text{-GPD cyt.}}{NAD^+\text{-GPD I+II}}$ (%)
7th h darkness	control	43.3	39.4
	+NAD^+	45.1	26.7
	+$NADP^+$	57.4	36.0
6th h light	control	61.1	34.0
	+NAD^+	42.9	39.1
	+$NADP^+$	51.0	18.2
DCMU	control	60.7	36.8
	+DCMU	44.3	26.8
Etiolated cotyledons		31.7	79.3


A) CONTROL EXTRACT
B) NAD+ EXTRACT
C) NADP+ EXTRACT
NADP+-GPD II
NAD+-GPD II
NADP+-GPD I
NAD+-GPD I
μ MOL NADP(H) OXIDIZED MIN-1
% (NH4)2 SO4 SATURATION
FRACTION NUMBER

tract and in slightly increased NAD^+-GPD I activity for the $NADP^+$ extract (result not shown).

The chloroplast ($NADP^+$-GPD I/$NADP^+$-GPD II) and the "cytosolic" (NAD^+-GPD I cyt./NAD^+-GPD I+II) activity ratios were computed from the calculated area under each isozyme peak. The chloroplast activity ratio (Tab. 1) was characterized by high values for the light plant control and all extracts using $NADP^+$ with a mean value ($\pm$SE) of 58.9 ± 2.4. Low values (43.2 ± 2.1) were obtained for the dark control plants and all extracts using NAD^+. The "cytosolic" activity ratio was found to be almost constant (37.1 ± 3.2) in all cases, except when NAD^+ was added to the dark extracts or $NADP^+$ added to the light extracts (Tab. 1).

The extracts from DCMU-treated leaves sampled at the end of the light period (Fig. 2) showed decreased GPD I isozyme activities as compared to non treated leaves (control extracts). This resulted in a chloroplast activity ratio (Tab. 1) similar to that observed in non treated leaves sampled during darkness. The cytosolic activity ratio was similar to that obtained with the NAD^+ extracts from dark plants.

Compared to those from light-grown material, the isozyme activities from etiolated cotyledons (Fig. 3) showed a very high NAD^+-GPD I peak height, some $NADP^+$-GPD I activity but no defined peak and reduced GPD II activities. The resulting chloroplast activity ratios (Tab. 1) were 25% lower than those obtained from green leaves sampled in darkness. The cytosolic activity ratio was twice greater than that observed from primary leaves.

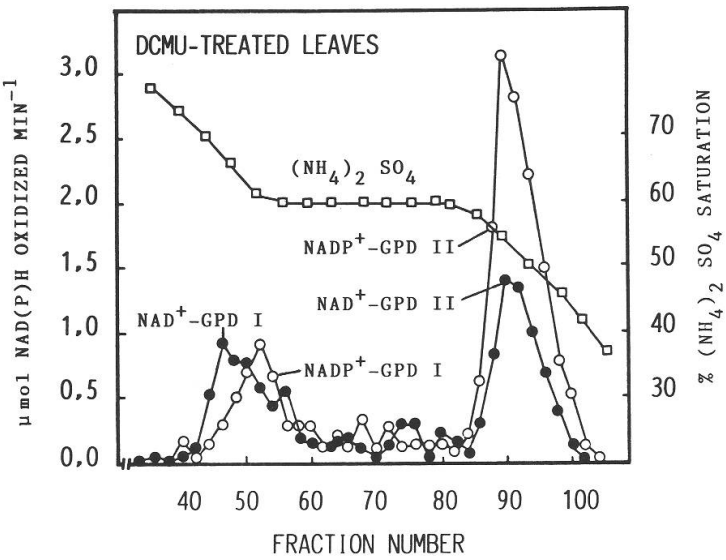


Fig. 2. Inverse ammonium sulphate gradient solubilization of the glyceraldehyde-3-phosphate dehydrogenase isozymes from 4-week old DCMU-treated primary spinach leaves.

Fig. 1. Effect of added NAD^+ and $NADP^+$ at the moment of extraction on the glyceraldehyde-3-phosphate dehydrogenase isozyme activities from 4-week old primary spinach leaves sampled after 7 h of darkness in a 8 h light:16 h dark cycle. Patterns obtained by inverse ammonium sulphate gradient solubilization of leaf extracts. A) control extract, B) and C) extracts with 0.2 mM NAD^+ and $NADP^+$, respectively.

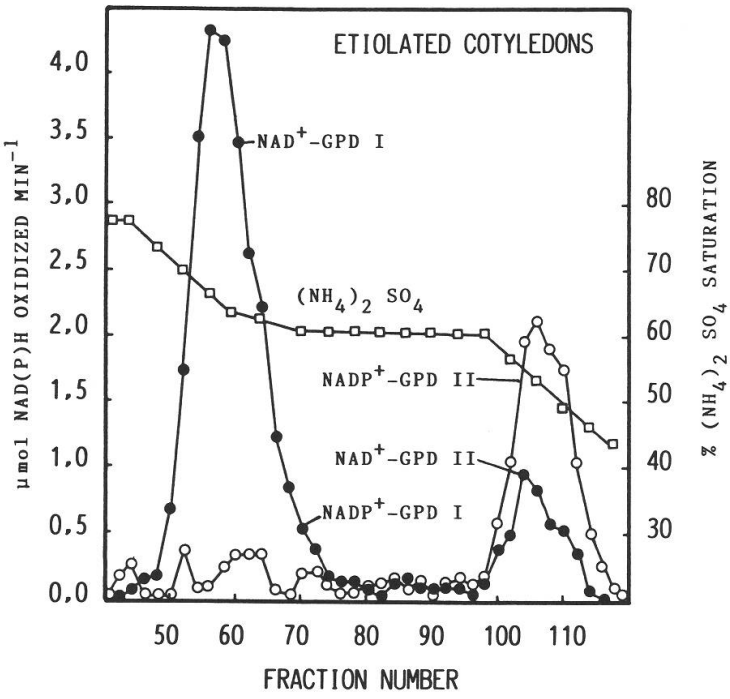


Fig. 3. Inverse ammonium sulphate gradient solubilization of the glyceraldehyde-3-phosphate dehydrogenase isozymes from 10-day old etiolated spinach cotyledons.

Discussion

In vitro modulation of the GPD isozyme activities by NAD^+ or $NADP^+$ at the moment of extraction (Fig. 1) was dependent on the physiological state of the leaves, i.e. on the sampling time in the light: dark cycle. The NAD^+ and $NADP^+$ extracts showed, if compared to the appropriate control extracts, that the GPD I activities could be modulated by NAD^+ in the light and by $NADP^+$ in darkness. This was indicating that the added pyridine nucleotides only modulate these activities when their respective endogenous concentrations are low (Bonzon et al. 1983). In contrast to this, the GPD II activities were only sensitive to added pyridine nucleotides in the dark: the NAD^+-GPD II and $NADP^+$-GPD II activities being increased by NAD^+ and $NADP^+$, respectively.

These effects were reflected in the $NADP^+$-GPD I/$NADP^+$-GPD II chloroplast activity ratio (Tab. 1). The dark control values could be mimicked by NAD^+ added to light extracts, whereas low values were obtained with $NADP^+$ added to the dark extracts. The influence of the $NAD^+/NADP^+$ ratio at the moment of extraction was also reflected in the cytosolic activity ratio. The low value for the NAD^+ dark extract was due to the high NAD^+-GPD II (chloroplastic) activity, whereas the low value for the $NADP^+$ light extract seemed to be due to a decreased NAD^+-specific cytosolic activity.

The activities obtained from DCMU-treated leaves (Fig. 2) showed decreased GPD I activities. The low cytosolic activity ratio, which might be due to a decreased NAD^+-specific cytosolic activity, suggests that only the chloroplast GPD I isozymes are activated by the photosynthesis-derived changes in the redox state (Müller et al. 1969; Wolosiuk and Buchanan 1977; Lendzian and Ziegler 1978).

In etiolated cotyledons (Fig. 3), the very high NAD^+-GPD I activity was characteristic of plants having high glycolytic activities such as light-grown *Chenopodium rubrum* seedlings in the presence of glucose, or the photosynthesis inhibitor SAN 9789 (de Looze 1980). This was a strong indication that this activity is mainly due to the NAD^+-specific cytosolic enzyme. The low $NADP^+$-GPD I activity indicates that there might be some NAD^+-GPD I "chloroplastic" activity in the corresponding peak and, together with the GPD II activities, it might allow one to infer that the two bifunctional chloroplast isozymes are present in the etioplast.

In relation to the different experimental conditions tested in this study, the observed GPD activity changes reflected the sensitivity of the GPD isozymes to in *vitro* modulation by physiologically or near *in vivo* situations of redox state and by environmental and chemical factors. The NAD^+ and $NADP^+$ effects, when compared to the situation in etiolated cotyledons and in DCMU-treated leaves, showed differences in correlation with potential *in vivo* modulation of nucleotide ratios (Hampp et al. 1982). This modulation of the isozyme activities in response to a change in the cellular $NAD^+ / NADP^+$ ratio was also an indication supporting the interpretations mentioned in the introduction.

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