Effects of photoperiod and plant developmental stage on NADH dehydrogenase and photosystem activities of isolated chloroplasts

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Abstract

Barley (*Hordeum vulgare* L. cv. Hassan) leaves were used to study the effects of developmental stage and photoperiod on the NAD(P)H-ferricyanide oxidoreductase (NAD(P)H-FeCNR) and on the photosystem (PS) 1 and 2 activities of isolated chloroplasts. From day 6 to day 12, both the PS 1 and NADH-FeCNR activities decreased while NADPH-FeCNR activity remained almost unchanged. Methyl jasmonate had no significant effect on the NAD(P)H-FeCNR activity changes. In 6- to 7- and 14- to 15-d-old plants, the NADH-FeCNR activity was higher during the photoperiod than during the dark period and, in the 14- to 15-d-old plants, the PS 1 activity increased during photoperiod in the same way, but to a lesser extent. The PS 1 activity of plants during a dark-accelerated senescence was low. The simultaneous changes in chloroplast PS 1 and NADH-FeCNR activities support the role proposed for the chloroplast NADH dehydrogenase complex in the cyclic electron transport.

Additional key words: barley, *Hordeum vulgare*, light stimulation, methyl jasmonate, NAD(P)H-ferricyanide oxidoreductase.

Introduction

Chloroplasts of higher plants contain an NAD(P)H dehydrogenase (NAD(P)H DH) complex (Sazanov *et al.* 1998) that is homologous to the NADH-ubiquinone oxidoreductase (complex I; EC 1.6.5.3) of bacteria and mitochondria (Friedrich *et al.* 1995). This enzyme is composed of at least eleven subunits (NDH-A to K) encoded in the plastid genome (Friedrich *et al.* 1995). The chloroplast NAD(P)H DH complex has been isolated and partially characterized (Quiles *et al.* 1996, Sazanov *et al.* 1996, 1998, Quiles and Cuello 1998). It is located in stroma thylakoids (Berger *et al.* 1993, Sazanov *et al.* 1996, Quiles *et al.* 1999).

However, the function of the chloroplast complex has not yet been clarified, although four possible functions have been proposed. Firstly, it may be involved in chlororespiration, a process extensively studied in unicellular green algae (Bennoun 1982, 1994, Peltier *et al.* 1987) but less so in higher plants (Garab *et al.* 1989, Gruszecki *et al.* 1994, Feild *et al.* 1998). The breakdown of saccharides in chloroplasts in the dark produces reduction equivalents (Singh *et al.* 1992), which would be oxidized by the chloroplast NAD(P)H DH. Secondly, the enzyme may be a component of the cyclic electron transport pathway around photosystem 1 (PS 1) (Burrows *et al.* 1998, Shikanai *et al.* 1998). This function possibly mediates chloroplast photoprotection (Endo *et al.* 1999). Moreover, the chloroplast NAD(P)H DH complex is associated to ferredoxin-$NADP^+$ oxidoreductase (EC 1.18.1.2) (Guedeney *et al.* 1996, Quiles and Cuello 1998, Funk *et al.* 1999), which is an electron acceptor of PS 1. Thirdly, the enzyme may be involved in the protection of chloroplasts against photooxidative stress, particularly in senescent leaves (Martin *et at.* 1996) and methyl jasmonate (MJ) increases

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Abbreviations: Chl - chlorophyll; MJ - methyl jasmonate; NAD(P)H DH - NAD(P)H dehydrogenase; NAD(P)H-FeCNR - NAD(P)H-ferricyanide oxidoreductase; PS - photosystem.

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the NADH DH activity of senescent leaf segments (Cuello *et al.* 1995b). Finally, the enzymatic complex may serve to generate a proton gradient across the prothylakoid membrane, which is necessary for protein integration into the membrane at developmental stages when a functional photosynthetic electron transport chain is not yet operating (Fischer *et al.* 1997).

As a contribution to knowledge of the function of the chloroplast NAD(P)H DH complex, we investigated the effects of the plant developmental stage and of the daily photoperiod on the PS 1, PS 2, and NAD(P)H-ferricyanide oxidoreductase (NAD(P)H-FeCNR) activities in isolated chloroplasts. We also investigated the possible effects of application of MJ to barley plants on the NAD(P)H-FeCNR activities.

Materials and methods

Plants: Barley (*Hordeum vulgare* L. cv. Hassan, supplied by INDACSA, Spain) was grown in a *Fisons 140 G-2* culture chamber (temperature 24 °C, 18-h photoperiod, irradiance *ca.* 15 W m^{-2}) or in *Selecta Hotcold GL* culture chamber (under lower irradiance of 7 W m^{-2}) as described previously (Cuello *et al.* 1987). From day 6 the plants were sprayed every 12 h with 45 μM MJ or distilled water (control). All experiments were carried out using the recently detached oldest leaf of the plants, after discarding the base and tip.

Isolation of chloroplasts: To measure NAD(P)H-FeCNR activities, 6 g of leaf segments were washed with distilled water and homogenized at 0 - 5 °C for 10 s in a *Sorvall Omni-mixer* in freshly prepared buffer (E) that contained 0.35 M sucrose, 25 mM Na-Hepes, 2 mM Na_2-EDTA, and 8 mM mercaptoethanol (pH 7.6). The homogenate was strained through 4 layers of muslin and centrifuged at 200 *g* for 5 min. The supernatant was centrifuged at 2 500 *g* for 10 min and the pellet was washed with buffer E. As reported previously (Cuello *et al.* 1995a), the chloroplast preparation was essentially free from mitochondria and other cellular components. Chloroplasts were then purified through sucrose gradient (Douce and Joyard 1982), during which they lost most NADH-FeCNR activity (results not shown), while the intact chloroplast percentage of the preparations was at least 70 %. The chloroplasts were finally resuspended and lysed in 50 mM Tris-HCl buffer (pH 8.0) to 350 μg(Chl) cm^{-3}. Before measuring the activity, the suspension of broken chloroplasts was treated with 0.4 % (m/v) sodium deoxycholate for 45 min.

To measure PS activities, the chloroplasts were isolated as before except that buffer E contained 0.5 mM Na_2-EDTA and 2 mM ascorbic acid (instead of mercaptoethanol) and the chloroplasts were resuspended in a buffer R containing 50 mM N-[2-hydroxyethyl] piperazine-N'-[3-propanesulfonic acid]-KOH, 50 mM sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$ (pH 7.8) up to 150 μg(Chl) cm^{-3} and kept at 4 °C in the dark until use.

Enzyme and PS 1 and PS 2 assays: The NAD(P)H DH activities were determined with potassium ferricyanide as electron acceptor. The NAD(P)H-FeCNR activities were determined in a *ATI* (*Unicam* UV spectrophotometer, Cambridge, UK) at 25 °C, as described by Galante and Hatefi (1979), by continuously measuring the decreases in A_{340} which were due to oxidation of NAD(P)H. The reaction mixture, with a final volume of 1 cm^3, included 50 mM potassium phosphate (pH 8.0), 1 mM Na_2-EDTA, 0.15 mM NADH or NADPH, 1 mM potassium ferricyanide, and chloroplast sample (6.75 or 13.50 μg Chl and 30.37 or 60.75 μg protein) for NADPH- and NADH-dependent activities, respectively. Absorbance decreases were recorded after addition of the enzyme sample and the linear decrease, proportional to the amount of enzyme, was determined between 10 and 70 s of the reaction. The control values, obtained from media without enzyme preparation, were subtracted. The enzymatic activity was expressed as nmol of NAD(P)H oxidized per mg Chl, mg protein or g leaf fresh mass per s in the reaction conditions.

The PS 2 and PS 1 activities were measured independently and spectrophotometrically in assay mixtures as described by Xiao *et al.* (1997), except that these were exposed to saturating irradiance (700 W m^{-2}) for 30 s at 30 °C, using the washed chloroplasts resuspended in buffer R. In the above conditions, proportionality between Chl concentration and PS activity was found up to an A_{580} increase of 0.25 per min. PS activity was expressed as nmol of 2,6-dichlorophenol indophenol oxidized (for PS 1) or reduced (for PS 2) per mg Chl or g leaf f.m. per s in the reaction conditions.

Protein was quantified using the method of Lowry *et al.* (1951) after precipitation with 10 % (m/v) trichloroacetic acid. Chl content was determined according to Arnon (1949).

Results

Effect of plant developmental stage on PS 1, PS 2, and NAD(P)H-FeCNR activities: The plants used in this study were grown under irradiance of 7 W m^{-2} and the leaves were always harvested 11 h after the beginning of the photoperiod. In the control plants, the NADH-FeCNR activity decreased steadily from day 6 to day 12, and increased slightly in 14-d-old plants. In contrast, the NADPH-FeCNR activity did not change very much with plant growth, although in 8- and 10-d-old plants the activity seemed to rise somewhat transitorily. MJ applied had very little effect, if any, on the NAD(P)H-FeCNR activity levels (Fig. 1).

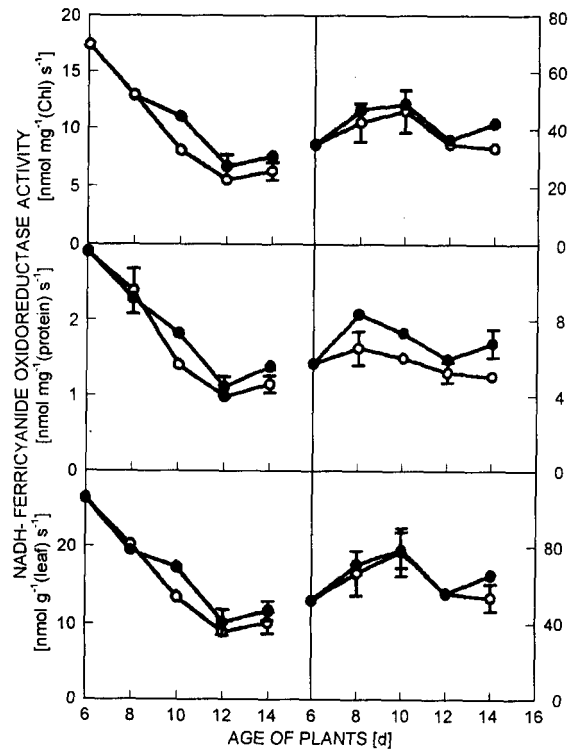

Fig. 1. Effect of applied methyl jasmonate (*closed symbols*) on the NADH-FeCNR and NADPH-FeCNR activities measured in chloroplasts isolated from 6- to 14-d-old plants grown under irradiance of 7 W m^{-2}. Effect of distilled water (control) - *empty symbols*. Means from 4 - 6 independent experiments. Only SE ≥ 10 % are presented.

To confirm the functional relationship between the chloroplast NADH DH complex and the PS 1 in the cyclic photosynthetic electron transport, the effects of the plant age on PS 1 and PS 2 activities were studied. These assays included 14-d-old plants grown under irradiance 7 W m^{-2} during 18-h photoperiod and then transferred to continuous darkness for 4 d. This dark period induced a significant increase, at least on Chl basis, in NADH-FeCNR activity (results not shown). PS 1 activity strongly diminished from day 6 to day 14, whereas PS 2 activity fell very little (Fig. 2). However, when 14-d-old plants grown under photoperiod were transferred to the dark, PS 1 activity remained constant whereas PS 2 activity fell sharply (Fig. 2).

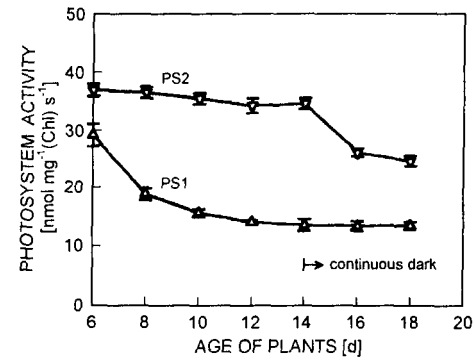

Fig. 2. Photosystem 1 and 2 activities during the barley plant development. Plants grown under 18-h photoperiod with irradiance of 7 W m^{-2} were transferred at day 14 to continuous darkness for a further four days. Means ± SE from 5 - 6 independent assays.

Daily courses of PS 1, PS 2, and NAD(P)H-FeCNR activities: For this study, leaves of plants grown under irradiance of 15 W m^{-2} were harvested at the end of the 18-h light period or 6-h dark period. The NADH-FeCNR activity at the end of light period was higher than at the end of dark period in both 6- and 14-d-old plants (Table 1). The smaller recovery of the NADH-FeCNR activity during the following photoperiod in 7-d-old plants might to be due, at least in part, to the decrease in activity due to ageing (Fig. 1). On the other hand, in our assay conditions, the chloroplast NADPH-FeCNR activity was less affected by the light or dark period in the two developmental stages studied (Table 1).

Within the changes induced by the light-dark cycle, we studied the possible simultaneous changes in PS 1 and PS 2 activities. Since the PS 1 activity decreased too quickly with increasing age between day 6 and 7 (Fig. 2) effect of day time on PS activities was studied only in 14- and 15-d-old plants (Table 2). The PS 1 activity at the end of dark period was lower than the activity in previous and following light period (Table 2). In contrast, the slight diminution in PS 2 activity during the dark period was not recovered during the following light period (Table 2).

Table 1. Effects of the light and dark period on chloroplast NAD(P)H-FeCNR activities. Plants were grown under 18-h photoperiod with irradiance of 15 W m^{-2} and the chloroplasts were isolated from 6- and 14-d-old plants at the end of the light period (L1) and dark period (D1), and from 7- and 15-d-old plants at the end of the following light period (L2). Means ± SE from 4 - 8 independent experiments. The activities were expressed in nmol mg^{-1}(Chl) s^{-1} (Chl), nmol mg^{-1}(protein) s^{-1} (protein), or nmol g^{-1}(leaf f.m.) s^{-1} (leaf).

Age [d]	Period	NADH-FeCNR			NADPH-FeCNR		
		Chl	protein	leaf	Chl	protein	leaf
6	L1	19.16 ± 0.66	2.83 ± 1.66	27.00 ± 1.33	50.00 ± 2.66	7.33 ± 0.50	70.50 ± 4.66
6	D1	10.66 ± 1.50	1.50 ± 1.66	14.83 ± 2.50	52.00 ± 2.16	7.16 ± 0.00	72.16 ± 4.33
7	L2	15.66 ± 1.16	2.16 ± 1.66	24.00 ± 2.16	50.66 ± 4.33	7.66 ± 0.50	77.50 ± 7.83
14	L1	7.50 ± 0.33	1.33 ± 0.00	12.66 ± 0.83	46.83 ± 2.66	7.83 ± 0.50	79.16 ± 5.83
14	D1	4.33 ± 1.66	0.83 ± 0.00	7.50 ± 0.50	42.83 ± 2.33	7.50 ± 0.33	72.83 ± 5.16
15	L2	7.16 ± 0.33	1.16 ± 0.00	12.00 ± 0.83	45.16 ± 3.83	7.00 ± 0.66	77.33 ± 8.00

Table 2. Effects of the light and dark period on photosystem activities. Plants were grown under 18-h photoperiod with irradiance of 15 W m^{-2} and the chloroplasts were isolated from 14-d-old plants at the end of the light period (L1) and dark period (D1), and from 15-d-old plants at the end of the following light period (L2). Means ± SE from 6 independent experiments. The activities were expressed in nmol mg^{-1}(Chl) s^{-1} (Chl) or nmol g^{-1}(leaf f.m.) s^{-1} (leaf).

Period	PS1		PS2	
	Chl	leaf	Chl	leaf
L1	14.66 ± 0.83	24.83 ± 1.66	42.50 ± 1.16	71.66 ± 3.00
D1	13.33 ± 0.66	22.66 ± 1.66	38.33 ± 0.66	65.33 ± 2.33
L2	14.66 ± 0.66	24.50 ± 1.66	39.16 ± 1.83	65.50 ± 4.50

Discussion

The NADH DH complex has NADH-FeCNR activity (Quiles and Cuello 1998). However, the variation in the NAD(P)H-FeCNR activities cannot be attributed solely to the NADH DH complex, and other chloroplast enzymes may contribute to these activities. As far as we know only ferredoxin-$NADP^+$ oxidoreductase has been reported as a chloroplast enzyme with diaphorase activity *in vitro*, but whereas it is probable that most of the NADPH-FeCNR activity measured in chloroplasts was due to the diaphorase activity of ferredoxin-$NADP^+$ oxidoreductase (Cuello *et al.* 1995a, Sazanov *et al.* 1995), this cannot be so in the case of NADH-FeCNR activity because NADH is a very poor electron donor for ferredoxin-$NADP^+$ oxidoreductase (Sancho *et al.* 1988, Cartagena *et al.* 1994).

The high decrease in NADH-FeCNR activity and the relative stability of NADPH-FeCNR activity during the first stages of barley growth (Fig. 1) might be explained, at least in part, by different biosynthetic profiles of both the NADH DH complex and ferredoxin-$NADP^+$ oxidoreductase during plant development. The highest values of NADH-FeCNR activity were found in 6-d-old plants (Fig. 1), which suggests some function for the NADH DH complex from very early stages of plant development. These results agree with those of Fischer *et al.* (1997), who found high amounts of NDH-H and NDH-K in etioplasts and observed that these proteins were present during the youngest stages of chloroplast development. However, the fact that RNA editing of *ndh*D is suppressed to a very low level in non-photosynthetic cells and activated by light in tobacco leaves indicates that the gene product must function in photosynthetic cells in the light (Hirose and Sugiura 1997). Catalá *et al.* (1997) found that during leaf ontogeny, the NDH-F content decreased from young to mature tissue but increased during senescence. Not discarding the role of the NADH DH complex in protein integration into the prothylakoid membrane (Fischer *et al.* 1997), the simultaneous decreases of NADH-FeCNR (Fig. 1) and PS 1 (Fig. 2) activities during plant development support the role of the NADH DH complex participating in the cyclic photosynthetic electron transport around PS 1 (Burrows *et al.* 1998, Shikanai *et al.* 1998), and agree with the importance of the energy supply by cyclic photophosphorylation in developing chloroplasts (Fischer *et al.* 1997). The photosynthetic function of the complex is connected with the location of subunits of the enzyme complex in the stroma thylakoids

(Berger *et al.* 1993, Sazanov *et al.* 1996, Quiles *et al.* 1999), where the cyclic transport occurs. However, the constant low PS 1 activity observed after transferring 14-d-old plants grown under photoperiod to the continuous dark (Fig. 2) and the increased NADH-FeCNR activity observed at the same time (results not shown) suggest a function other than photosynthetic electron transport for the NADH DH complex during leaf senescence (Cuello *et al.* 1995b). Such a function might be related with the stimulatory effect of MJ on the NADH-FeCNR activity in darkened leaf sections (Cuello *et al.* 1995b), and the negligible effect of MJ on attached leaves growing under photoperiod (Fig. 1).

If the NADH DH complex participates in photosynthesis, it is probably activated by light, similarly as other photosynthetic enzymes. The NADH-FeCNR activity increased cyclically during the daily photoperiod in both young and adult plants (Table 1). Moreover, results not shown indicate that all the activation of NADH-FeCNR from 7-d-old plants occurs within one hour after the beginning of the light period and the inactivation within one hour after the beginning of the dark period. The possible light activation of the NADH DH complex was already suggested (Cuello 1997); this activation could be a consequence of the photosynthetic role of the complex. According to Teicher and Scheller (1998) the NAD(P)H DH complex of barley thylakoids is activated by light, but they used NADPH as electron donor. In any case, the preceding irradiation of plants may affect the NADH DH activity of chloroplasts isolated from them. Although to a lesser extent than NADH-FeCNR activity, PS 1 activity also cyclically increased during the light period (Table 2), as might be expected if the NADH DH complex participates in the cyclic electron transport around PS 1. Radiant energy seemed to have a differential activating effect on NADH DH and ferredoxin-$NADP^+$ oxidoreductase since in the young and adult plants only the NADH-FeCNR activity was strongly and cyclically stimulated under the photoperiod (Table 1). The apparent lack of effect of the daily period on NADPH-FeCNR activity (Table 1) was probably due, at least in part, to the dark-provoked inactivation of ferredoxin-$NADP^+$ oxido-reductase, which can occur in a few minutes during chloroplast isolation (Satoh 1981).

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