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The NAD(P)H Dehydrogenase in Barley Thylakoids Is Photoactivatable and Uses NADPH as well as NADH[1]

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An improved light-dependent assay was used to characterize the NAD(P)H dehydrogenase (NDH) in thylakoids of barley (*Hordeum vulgare* L.). The enzyme was sensitive to rotenone, confirming the involvement of a complex I-type enzyme. NADPH and NADH were equally good substrates for the dehydrogenase. Maximum rates of activity were 10 to 19 μmol electrons mg^{-1} chlorophyll h^{-1}, corresponding to about 3% of linear electron-transport rates, or to about 40% of ferredoxin-dependent cyclic electron-transport rates. The NDH was activated by light treatment. After photoactivation, a subsequent light-independent period of about 1 h was required for maximum activation. The NDH could also be activated by incubation of the thylakoids in low-ionic-strength buffer. The kinetics, substrate specificity, and inhibitor profiles were essentially the same for both induction strategies. The possible involvement of ferredoxin:$NADP^+$ oxidoreductase (FNR) in the NDH activity could be excluded based on the lack of preference for NADPH over NADH. Furthermore, thenoyltrifluoroacetone inhibited the diaphorase activity of FNR but not the NDH activity. These results also lead to the conclusion that direct reduction of plastoquinone by FNR is negligible.

The genomes of cyanobacteria and most plant chloroplasts contain 11 genes (*ndh*A-*ndh*K) with sequence similarity to the subunits of NADH dehydrogenase (complex I) (Berger et al., 1993). The *ndh* gene products form a complex that can be isolated from thylakoid membranes (Funk and Steinmüller, 1995; Sazanov et al., 1995; Quiles et al., 1996). In bacteria (other than cyanobacteria) complex I consists of 14 subunits that are also conserved in mitochondrial complex I (Friedrich et al., 1995). The cyanobacterial and chloroplast NDH seem to lack homologs of the three essential subunits that constitute the NADH-oxidation site in the bacterial and mitochondrial complex (Grohman et al., 1996).

The function of NDH in chloroplasts is not understood, but a role in cyclic electron transport and/or chlororespiration would seem to be likely. A role in cyclic electron transport would imply electron donation from stromal NADPH via the membrane-bound NDH complex to the plastoquinone pool. Kubicki et al. (1996) showed that in sorghum the *ndh* genes are preferentially expressed in bundle-sheath chloroplasts, the apparent site of cyclic electron flow in C_4 species. Thus, this finding is in agreement with a function of the NDH complex in cyclic electron transport.

NDH activity has been demonstrated in the thylakoid membranes of several different species of plants, algae, and cyanobacteria (Mi et al., 1992a, 1992b, 1994, 1995; Yu et al., 1993; Cuello et al., 1995; Sazanov et al., 1995; Quiles et al., 1996; Seidel-Guyenot et al., 1996). However, direct demonstration of an involvement in cyclic electron transport in most cases has not been achieved. Mi et al. (1995), working with *ndh* mutants of *Synechocystis* sp. PCC 6803, have presented the most clear evidence for an involvement of the NDH complex in cyclic electron transport. In contrast, *ndh* mutants of *Synechococcus* sp. PCC 7002 were not deficient in cyclic electron transport (Yu et al., 1993). Cyclic electron transport via NDH is most easily understood if the complex can use NADPH, as shown by Mi et al. (1995). However, there is a lack of consensus on the substrate specificity of the NDH complex. In barley (*Hordeum vulgare*) and in pea, NADH-specific oxidation has been reported (Cuello et al., 1995; Sazanov et al., 1995). Because the thylakoid NDH activity in plants has been studied in only a few instances, it is tempting to draw on studies in algae and cyanobacteria. In the unicellular algae *Chlamydomonas reinhardtii* and *Pleurochloris meiringensis*, which are both species with a high rate of chlororespiration in the dark, the dehydrogenase activity in the thylakoid membranes is most efficient with NADH (Godde and Trebst, 1980; Seidel-Guyenot et al., 1996). In cyanobacteria the situation is complex; whereas NADPH-specific activity has been reported in *Synechocystis* sp. PCC 6803 (Mi et al., 1995), different specificities have been reported for other species (for review, see Schmetterer, 1994).

It is futile to look for a unifying principle covering all oxygenic phototrophs. It seems likely that NDH is involved in both cyclic and respiratory pathways, and that its relative contribution to different pathways may differ between species or even within a species dependent on growth conditions. The issue of specificity is complicated by the different assay conditions used, by mitochondrial contamination, and by interference from NADPH oxidation by FNR. In most previous studies the dehydrogenase activities have been

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Abbreviations: Chl, chlorophyll; FNR, Fd:$NADP^+$ oxidoreductase; MeV, methyl viologen; NDH, NAD(P)H dehydrogenase; TTFA, thenoyltrifluoroacetone.

assayed in the dark with artificial acceptors such as ferricyanide or soluble quinones. Both mitochondrial complex I and FNR will show high activity in such assays. In this paper we have used a light-specific assay that eliminates the interference from contaminating activities, and we clearly demonstrate an NAD(P)H dehydrogenation that functions with equal efficiency with both substrates.

A further unsolved question is which subunit(s) contains the NAD(P)H-binding site of the NDH complex? The chloroplast genome has no homolog of the NADH-binding flavoprotein of complex I, and evidence against the presence of a nuclear-encoded chloroplast homolog has been presented (Grohman et al., 1996). The genome of *Synechocystis* sp. PCC 6803 contains open reading frames in the hydrogenase operon with some similarity to the NADH-oxidizing subunits in other bacteria (Appel and Schulz, 1996). Whether the gene products are part of cyanobacterial NDH remains to be shown. Quiles et al. (1996) reported the presence of a 53-kD NADH-oxidizing protein in barley chloroplasts, and have suggested that this protein could be a component of the NDH complex. The 53-kD protein was specific for NADH rather than NADPH. Guedeney et al. (1996) showed that the flavoprotein FNR binds to several polypeptides of the NDH in tobacco thylakoids, and have suggested that FNR in thylakoids could be the functional equivalent of the NADH-oxidizing domain in complex I. This could explain the result of Mi et al. (1995), who provided evidence that the *Synechocystis* sp. PCC 6803 mutant deficient in NDH was also deficient in Fd-catalyzed cyclic electron transport. In contrast to this result, cyclic electron transport of barley thylakoids could not be inhibited by antibodies against FNR (Scheller, 1996).

In this study we present a number of decisive arguments against the involvement of FNR in the NDH activity. We present evidence that confirms and characterizes the presence of a bispecific, NDH-dependent electron flow in barley, and address a number of conflicts that exist from previous publications in this field. Finally, we report that NAD(P)H dehydrogenation in isolated thylakoids of barley requires induction for maximal rates of activity. Induction can be triggered by brief illumination or by incubation of thylakoids in a low-ionic-strength buffer.

MATERIALS AND METHODS

Isolation of Thylakoids

Seedlings of barley (*Hordeum vulgare* L. cv Svalöf's Bonus) were grown at 20°C in vermiculite with a 12-h photoperiod. Fluorescent tubes provided a PPFD of 80 μmol m^{-2} s^{-1}. After 7 d the seedlings were harvested at the onset of the light period, and leaves were homogenized in a buffer of 0.4 M Suc, 10 mM NaCl, 5 mM $MgCl_2$, 10 mM Tricine (pH 7.5), and 10 mM sodium ascorbate using a blender equipped with razor blades. The chloroplasts were precipitated by centrifugation at 2000*g* for 10 min. The chloroplasts were resuspended and lysed in 5 mM Tricine (pH 7.9) and the thylakoids were precipitated at 10,000*g* for 10 min. The thylakoid pellet was resuspended at a concentration of 0.5 to 2 mg Chl/mL in homogenization buffer with 20% glycerol and no ascorbate. Thylakoid preparations used for the study of FNR and Cyt *c* oxidase activity had a protein:Chl ratio of about 5:1. The thylakoids were frozen in liquid N_2 and stored at −80°C. The thylakoids remained fully active in all of the pathways investigated for at least 6 months.

Linear and Cyclic Electron Transport

Linear electron flow in thylakoids was measured as rates of O_2 consumption, using a Clark-type O_2 electrode. The reaction medium contained (in a total volume of 3 mL): 33 mM Tricine (pH 7.5), 83 mM NaCl, 10 mM $MgCl_2$, 0.1 mM MeV, 6.7 mM NH_4Cl, 0.3 mM NaN_3, and thylakoids corresponding to 60 μg of Chl. Fd-catalyzed cyclic electron transport was measured as rates of $P700^+$ reduction under anaerobic and redox-poised conditions as described previously (Scheller, 1996).

Light-Dependent NAD(P)H Oxidation

Quantitation of light-dependent NAD(P)H oxidation was carried out by a modification of the procedure of Mi et al. (1995). The reaction mixture contained (in a total volume of 0.5 mL): 24 mM Tris-HCl (pH 8.6), 48 mM NaCl, 9.6 mM $MgCl_2$, 200 μM NAD(P)H, 100 μM MeV, 10 μM DCMU, and thylakoids corresponding to 4 to 8 μg of Chl. The oxidation of NAD(P)H at 25°C was followed at 340 nm in a spectrophotometer (DW-2000, Aminco, Urbana, IL) with a thermostated cuvette holder. The actinic light was supplied by a halogen lamp (15 V, 150 W; model KL1500, Schott, Cologne, Germany) provided with fiber optics and passed through a 665-nm cut-on filter. The photomultiplier tube was protected from the actinic light with a 340-nm narrow-bandwidth interference filter. The oxidation of NAD(P)H was calculated using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. Before spectrophotometric analysis, the reagents, except for NAD(P)H, were mixed and the NDH activity was activated by illumination. The sample was surrounded by a water-cooled jacket (25°C) and white light was supplied by a halogen lamp (KL1500 Schott) provided with fiber optics. Details of illumination times are given in "Results." Alternatively, activation was accomplished by omitting $MgCl_2$ from the reaction mixture, and incubating the sample for 1 h in the dark in the presence of 12 μM NADPH.

Light-Independent NAD(P)H Oxidation

The rate of light-independent NAD(P)H oxidation was measured spectrophotometrically at 340 to 375 nm (using an extinction coefficient of 4.0 mM^{-1} cm^{-1}) as the rate of NAD(P)H oxidation at 25°C in an assay mixture containing (in a total volume of 0.5 mL): 20 mM Tris-HCl (pH 7.8), 8 mM $MgCl_2$, 200 μM NAD(P)H, and 120 μM duroquinone. The reaction also contained thylakoids corresponding to 8 μg of Chl or 4 pmol of isolated barley FNR. The reaction was started by the addition of duroquinone.

Chemicals and Various Assays

NADPH and NADH were obtained from Sigma (highest grade available) or from Boehringer Mannheim. The use of high-grade pyridine nucleotides is essential because lower-grade reagents may be contaminated with inhibitors of NDH (Dalziel, 1963). All other reagents were of analytical grade.

Chl was determined according to the method of Arnon (1949), and Cyt *c* oxidase activity was measured according to the method of Jesaitis et al. (1977). Protein concentration was determined by the bicinchoninic acid procedure according to the method of Dunn (1989). Barley FNR was isolated essentially according to the method of Serrano and Rivas (1982). Free FNR and the proteins of the photosynthetic membrane were resolved by SDS-PAGE using 8 to 25% polyacrylamide gels. Western blotting was performed essentially according to the method of Andersen et al. (1992) by electrophoretic transfer of thylakoid proteins and free FNR to nitrocellulose membranes, incubation with polyclonal rabbit antibodies raised against FNR isolated from barley, and final incubation with secondary swine antibodies conjugated with alkaline phosphatase.

RESULTS

Thylakoids Support Light-Dependent Oxidation of NADPH and NADH

To analyze the NDH activity without interference from mitochondrial complex I or from the diaphorase activity of FNR, we used a light-dependent assay, in which NAD(P)H oxidation was driven by PSI in the presence of MeV. The NAD(P)H oxidation was completely dependent on light, and no background oxidation in the dark could be detected (Fig. 1). Furthermore, no NAD(P)H oxidation was observed in the absence of MeV (data not shown). In the experiment shown in Figure 1, the rate of NADPH oxidation was 15 ± 2 μmol electrons mg^{-1} Chl h^{-1} and the rate of NADH oxidation was 15 ± 3 μmol electrons mg^{-1} Chl h^{-1}. Whole-chain linear electron transport was determined to be about 450 μmol electrons mg^{-1} Chl h^{-1}, showing that the thylakoids had an intact PSI and intersystem chain. Fd-catalyzed cyclic electron transport in barley thylakoids in the presence of 25 μM reduced Fd was determined by Scheller (1996) to be 36 μmol electrons mg^{-1} Chl h^{-1}, i.e. 8% of whole-chain electron-transport rates or 2.5 times that of the thylakoid NDH activity.

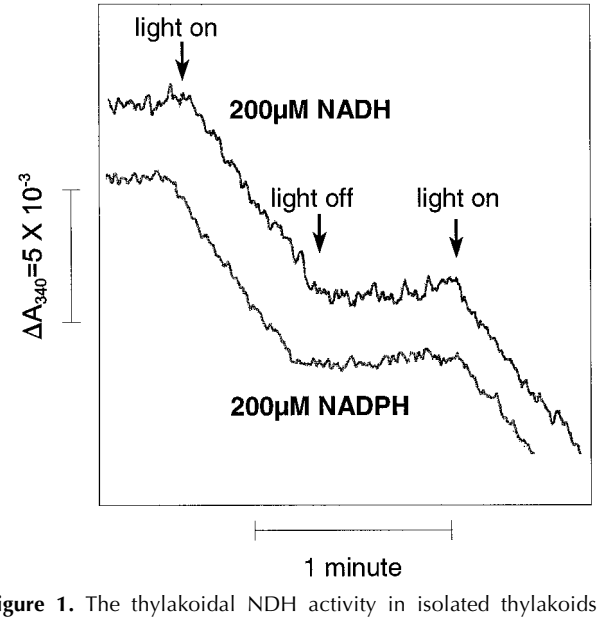

Figure 1. The thylakoidal NDH activity in isolated thylakoids of barley is NAD(P)H bispecific. The assay used in this study for measuring the oxidation of NAD(P)H with MeV as the electron acceptor is absolutely light dependent and thus specific for thylakoidal NDH activity. The NDH was activated by illuminating the thylakoids in the presence of the assay reagents for 45 min before measurement.

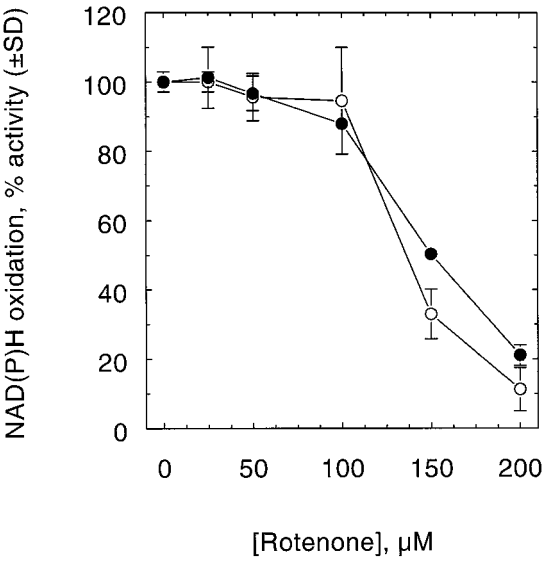

Figure 2. Inhibition of light-dependent NADPH (○) and NADH (●) oxidation by rotenone in isolated thylakoids. The thylakoids were activated by preillumination for 45 min. The activity of thylakoids in the absence of rotenone was 19 μmol electrons mg^{-1} Chl h^{-1} for the oxidation of NADPH and 20 μmol electrons mg^{-1} Chl h^{-1} for the oxidation of NADH.

Even though the light-dependent assay eliminates the interference from mitochondrial complex I, we resolved to quantitate the degree of mitochondrial contamination. Cyt *c* oxidase activity was measured on whole-leaf homogenate, on the supernatant after centrifugation of the homogenate at 2000*g*, and on isolated thylakoid membranes. Expressed on the basis of total volume, the whole-leaf homogenate was capable of oxidizing 8.2 μmol Cyt *c*/min, the supernatant retained an activity of 5.8 μmol Cyt *c*/min, and the thylakoid membranes had an activity of 0.5 μmol Cyt *c*/min, representing a contamination of the thylakoid fraction by mitochondrial protein of 7%.

Rotenone is a classic inhibitor of mitochondrial complex I, acting on the ubiquinone-reducing Fe-S centers (Trumpower, 1981). Inhibition of light-dependent NAD(P)H oxidation by rotenone (inhibitor concentration at 50% inhibition ≈ 150 μM) confirms that light-dependent NAD(P)H oxidation is mediated by a complex I-type enzyme, i.e. NDH

(Fig. 2). This experiment also confirmed the similarity with the NADH- and NADPH-dependent activities. Antimycin A (20 μM) did not inhibit the light-dependent oxidation of NADPH.

FNR Is Not Involved in the Light-Dependent NADPH Dehydrogenation

TTFA is an inhibitor of mitochondrial electron transport, acting on succinate dehydrogenase (Trumpower, 1981). The light-dependent NADPH dehydrogenase activity was not inhibited by TTFA (Fig. 3). TTFA also did not inhibit the light-dependent NADH oxidation (data not shown). In contrast, TTFA inhibited duroquinone-mediated diaphorase activity of isolated FNR, as well as the light-independent, duroquinone-mediated NADPH oxidation by thylakoids (Fig. 3). To address further the issue of the involvement of FNR in the NDH activity we determined the amount of FNR in the thylakoids. Immunoblots of thylakoids and isolated FNR incubated with antibodies against FNR revealed that thylakoid fractions contained 5.8 nmol membrane-bound FNR/mg Chl (data not shown). In the light-independent assay with duroquinone, the thylakoids oxidized NADPH at a rate of 36 ± 7 μmol electrons mg^{-1} Chl h^{-1}, whereas isolated FNR oxidized at a rate of 91 ± 9 μmol electrons $nmol^{-1}$ h^{-1}. Based on an observation by Nielsen et al. (1995) that free FNR is about 10 times more effective than bound FNR in mediating the oxidation of NADPH by duroquinone, we can estimate that the thylakoid-bound FNR should be able to mediate NADPH oxidation at a rate of about 47 μmol electrons mg^{-1} Chl h^{-1}. Thus, the amount of FNR in the thylakoids can account for all of the NADPH oxidation observed in the duroquinone assay.

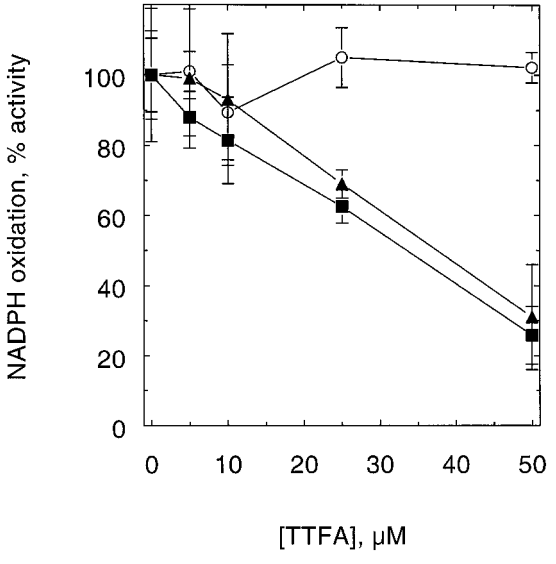

Figure 3. TTFA as an inhibitor of NADPH oxidation by isolated thylakoids or by FNR. The activity (± SD) was determined as light-dependent NADPH oxidation by thylakoids in the presence of MeV (○), as NADPH oxidation in the dark by thylakoids in the presence of duroquinone (▲), or as NADPH oxidation by isolated FNR in the presence of duroquinone (■). The light-dependent assay was performed with thylakoids activated by preillumination for 45 min. The activity of thylakoids in the absence of TTFA was 15 ± 2 μmol electrons mg^{-1} Chl h^{-1} for the light-dependent assay and 36 ± 7 μmol electrons mg^{-1} Chl h^{-1} for the duroquinone-dependent assay. The activity of FNR in the absence of TTFA was 91 ± 10 μmol electrons $nmol^{-1}$ FNR h^{-1}.

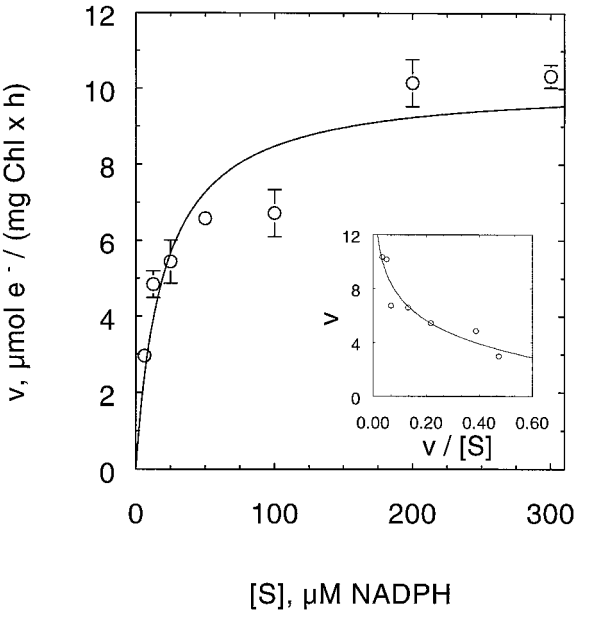

Figure 4. Thylakoidal NDH activity (± SD) as a function of NADPH concentration. The thylakoids were activated by preillumination for 45 min. The curve shows a fit obtained by hyperbolic regression. Inset, Eadie-Hofstee plot of the data.

Kinetic Properties of the NDH

Kinetic parameters were calculated for the light-activated oxidation of NADPH by thylakoid preparations. The NDH activity did not exhibit pure Michaelis-Menten kinetics (Fig. 4). The Eadie-Hofstee plot calculated from the data in Figure 4 was hyperbolic rather than linear (Fig. 4, inset), suggesting the presence of two kinetically different enzymes capable of the independent oxidization of NADPH, a phenomenon previously reported for thylakoidal NAD(P)H oxidation in the chromophytic alga *Pleurochloris meiringensis* (Seidel-Guyenot et al., 1996). Enzyme kinetics can be approximated using hyperbolic regression as V_{max} = 10 μmol electrons mg^{-1} Chl h^{-1}, K_m = 20 μM NADPH; or, using the Lineweaver-Burk plot, as V_{max} = 9 μmol electrons mg^{-1} Chl h^{-1}, K_m = 13 μM NADPH. The experiment was carried out four times with essentially the same results. Based on an observation by Sazanov et al. (1995) that the stoichiometric ratio of NADH dehydrogenase to photosynthetic reaction centers in spinach chloroplasts is about 1:100, the turnover number (K_{cat}) of the NDH characterized in this paper was calculated to be about 130 s^{-1}. The calculated K_{cat}/K_m ratio of 10^7 M^{-1} s^{-1} indicates a relatively high kinetic proficiency as well as a high substrate affinity, and suggests an important role for this enzyme in mediating electron transport.

Photoactivation of NDH

The rate of NADPH oxidation observed in thylakoids was higher if the thylakoids had been illuminated before measurement. Maximal rates of activity were obtained approximately 30 to 60 min after the onset of the illumination period. However, the light pulse required to achieve maximal activation may be as short as 5 min, after which full activation may be accomplished in the dark (Fig. 5). All of the results presented above for the MeV-mediated oxidation of NAD(P)H were acquired after 45 min of light induction.

With the standard assay mixture containing 9.6 mM $MgCl_2$ and 48 mM NaCl, or with 240 mM NaCl, light was required for induction of activity (Fig. 6). Similar data were obtained when $CaCl_2$ was substituted for $MgCl_2$. However, after incubation for up to 60 min in a low-salt assay medium (48 mM NaCl), light was not required for induction of the activity.

DISCUSSION

An assay has been used that specifically permits the measurement of NAD(P)H oxidation via the thylakoid NDH in isolated thylakoids of barley. The absolute light dependence of the assay (Fig. 1) excludes interference from the mitochondrial NADH dehydrogenase. Using a series of inhibitors it has been possible to determine that NAD(P)H is oxidized via a rotenone-sensitive, complex I-type chloroplast NDH. Rotenone inhibition was observed at higher concentrations (Fig. 2) than that necessary to inhibit mitochondrial complex I (Slater, 1967; Singer, 1979), but were in good agreement with previous reports of rotenone inhibition of thylakoidal electron transport (Cuello et al., 1995; Seidel-Guyenot et al., 1996).

In this study we demonstrate that electron transport via the chloroplast NDH complex shows no NADPH/NADH

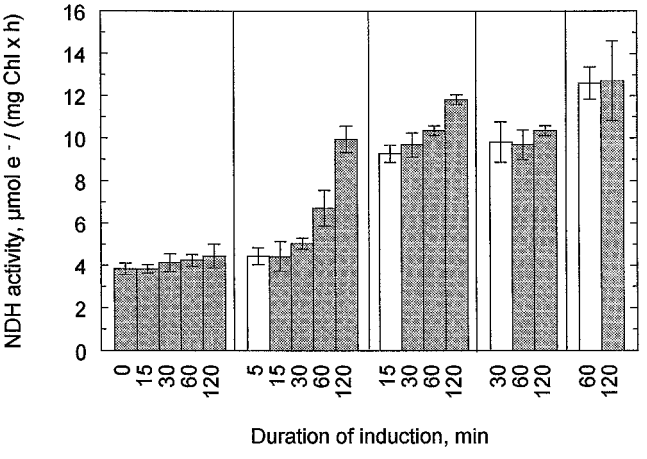

Figure 5. Light activation of the thylakoidal NDH. The thylakoids were illuminated in the presence of the assay reagents for the time periods indicated under the open bars. After illumination, the samples were kept in the dark and rates of NADPH oxidation (± SD) were determined at different time points. The numbers under the shaded bars indicate the total time from onset of the experiment, i.e. the sum of the illumination and subsequent dark periods.

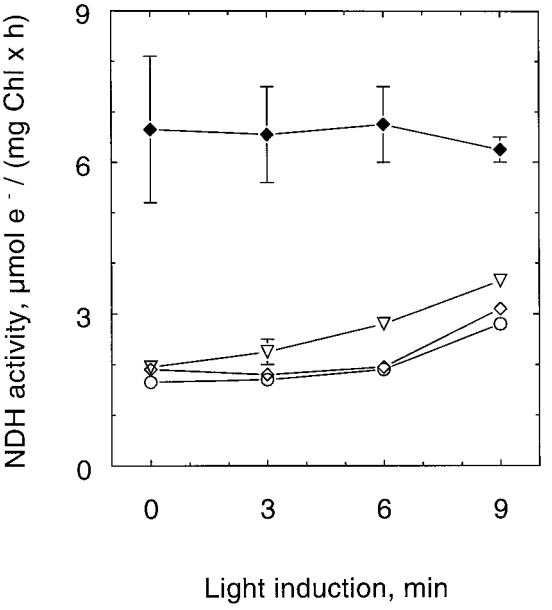

Figure 6. Induction of NDH activity. The assay mixtures contained low concentrations of monovalent cations: 48 mM Na^+ (◆); or high concentrations of mono- and/or divalent cations: 240 mM Na^+ (▽), 48 mM Na^+/9.6 mM Ca^{2+} (◇), or 48 mM Na^+/9.6 mM Mg^{2+} (○). The thylakoids were incubated in the dark in the presence of the respective assay reagents for 60 min, after which time the thylakoids were illuminated for the specified times before measurement of NADPH oxidation (± SD).

substrate specificity (Figs. 1 and 2). The identical inhibition of both NADH and NADPH oxidative activities of NDH by rotenone indicates that both activities are components of the same enzyme (Fig. 2). The fact that NADPH is a good substrate for the thylakoidal NDH strongly implies an involvement of this complex in cyclic electron transport. The NADPH/NADH bispecificity is an unusual (but not unique) property of an enzyme. The best-documented case for a bispecific NAD(P)H-dependent enzyme is an isoform of nitrate reductase (Campbell, 1996; Redinbaugh et al., 1996). The very similar enzymatic properties with both substrates observed with barley thylakoids, as reported in this paper, indicate that a bispecific NDH complex carries out the oxidation of both substrates. However, as the NAD(P)H-binding subunits of the NDH complex have still not been identified, one should be cautioned against premature conclusions. One possibility could be that the thylakoid NDH complex exists in two different forms distinguished by a different set of pyridine nucleotide-binding subunits. It can then be speculated that the plant may alter the relative content of the two forms dependent on the physiological requirements, e.g. for chlororespiration or cyclic electron transport. Such a scheme could explain the contradictory results obtained with different organisms.

NADH- and NADPH-oxidizing activities have been reported in chloroplasts of barley (Cuello et al., 1995) and pea (Sazanov et al., 1995), as well as in chloroplasts of *P. meiringensis* (Seidel-Guyenot et al., 1996), and it has been suggested that the NDH complex is specific for NADH, whereas the NADPH-oxidizing activity was mostly attrib-

utable to the diaphorase activity of FNR (Cuello et al., 1995; Sazanov et al., 1995). These workers have used a light-independent assay of thylakoidal NDH activity with ferricyanide or soluble quinones as the electron acceptor. Under these experimental conditions, FNR would be expected to exhibit a very high NADPH-oxidation activity (Cuello et al., 1995; Nielsen et al., 1995), as also demonstrated in the present investigation (Fig. 3). Thus, NADPH oxidation by the NDH complex may have been masked by FNR in the experiments by these workers.

Another source of NAD(P)H oxidation in our samples could be the activity of mitochondrial enzymes. We have determined the contamination of thylakoid preparations by mitochondrial proteins to be 7%. Using the values of Rasmusson and Møller (1991) we can calculate that with NADH as the substrate and O_2 as the acceptor, the mitochondrial enzymes could theoretically account for about 30 μmol electrons mg^{-1} Chl h^{-1} under our conditions, whereas NADPH oxidation would be negligible. However, we were not able to detect any oxidation of NADPH or NADH in the dark (Fig. 1), possibly because of the lack of Cyt *c* in our samples. This allows us to conclude that the contribution of mitochondrial NAD(P)H oxidation with O_2 as the electron acceptor in these samples is negligible.

The light-dependent, MeV-mediated oxidation of NADPH by barley thylakoids does not have a component of FNR-mediated activity. The NAD(P)H bispecificity of the thylakoidal NDH activity (Figs. 1 and 2) is convincing evidence against the involvement of FNR. Further evidence against the involvement of FNR is the insensitivity of MeV-mediated oxidation of NADPH to TTFA (Fig. 3). In addition, the K_m value of 13 μM NADPH for the light-dependent NADPH oxidation reported here differs significantly from the K_m value of 59 μM NADPH reported for the diaphorase activity of bound FNR (Nielsen et al., 1995). FNR efficiently catalyzes reduction of soluble quinones, and it has previously been speculated that FNR could directly mediate the reduction of plastoquinone in the membrane (Hosler and Yocum, 1985; Nielsen et al., 1995). However, no reduction by FNR of the physiologically more-relevant quinones, i.e. decyl-plastoquinone, vitamin K1, or ubiquinone-10, could be detected (Nielsen et al., 1995). If FNR could directly reduce plastoquinone, it should contribute to the MeV-dependent NADPH oxidation. Because no component of FNR-dependent activity was detectable, we conclude that direct reduction of membrane quinones by FNR must be negligible. Contrary to the light-dependent NDH activity, the light-independent NADPH oxidation activity appears to be similar to FNR diaphorase activity. Accordingly, it is most likely that light-independent NADPH oxidation in the presence of duroquinone is predominantly attributable to FNR bound to the thylakoid membrane. In summary, these results support the following three conclusions: (a) FNR does not interfere with the light-dependent assay for NDH activity, (b) FNR is not a component of the NDH complex in barley thylakoids, and (c) electron transfer from NADPH via FNR directly to plastoquinone must be very limited if it occurs at all.

The concentration dependency of the NDH activity indicates the presence of two enzymes (Fig. 4). The two activities appear to be saturated at 200 μM NADPH, i.e. the standard conditions for the light-dependent assay. Under these conditions no inhibition of activity by TTFA was observed (Fig. 3). Because TTFA strongly inhibits FNR, we can conclude that FNR does not make any detectable contribution to the activity shown in Figure 4. The indicated presence of a thylakoidal NDH activity derived from two enzymes more likely should be interpreted as the presence of a single enzyme in different states of activation, or as activities reflecting a modification to a fraction of the NDH complex pool.

The NDH-catalyzed NAD(P)H oxidation by light-induced thylakoid membranes was determined to be 10 to 19 μmol electrons mg^{-1} Chl h^{-1}. The variation in activities presented here may be attributable in part to the fact that the mechanism and requirements for activation of NDH activity are not yet fully understood. We believe that the rates reported here represent true physiological activities, and that previously published rates of NDH activity in plants have been overestimated because of interference from contaminating activities. Thus, in an assay measuring electron transport from NAD(P)H to potassium ferricyanide in barley (Cuello et al., 1995), the rate of NADPH oxidation can be calculated to be about 400 μmol electrons mg^{-1} Chl h^{-1}. However, as these workers point out, the specificity of the assay is such that much of the high rate with NADPH may be ascribed to the diaphorase activity of FNR. The rate of NADH oxidation in the same study can be calculated as 125 μmol electrons mg^{-1} Chl h^{-1}. Ferricyanide, however, is a much more efficient acceptor of electrons than the more natural acceptor, plastoquinone-1 (Godde, 1982). When this factor is taken into account, the rate of NADH oxidation found by Cuello et al. (1995) is comparable to the rate presented here.

The NDH activity reported here corresponds to about 3% of linear electron- transport rates. Fd-dependent cyclic electron transport corresponds to about 5% of linear electron transport, depending on the exact redox conditions (Scheller, 1996). Combined activities of cyclic electron transport of less than 10% of linear electron transport agree with cyclic electron-transport rates determined in microorganisms in vivo (Maxwell and Biggins, 1976; Myers, 1987; Yu et al., 1993; Ravenel et al., 1994).

The activation of NADPH-dependent electron flow through the thylakoidal NADPH dehydrogenase is shown to be light dependent (Fig. 5). The initial light-dependent step in the activation process may be related to the generation of ion gradients or oxidation of a redox mediator. Apparently, this initial step triggers a slower activating step. In the isolated thylakoid membranes, activation by phosphorylation or by thioredoxin cannot take place. One possibility would be activation by dephosphorylation. Redinbaugh et al. (1996) have reported activation by dephosphorylation of nitrate reductase, another bispecific pyridine-nucleotide dehydrogenase.

When the ionic strength is low, light is not required to trigger the activation, whereas rates of NDH activity are generally lower for ionically activated than for photoacti-

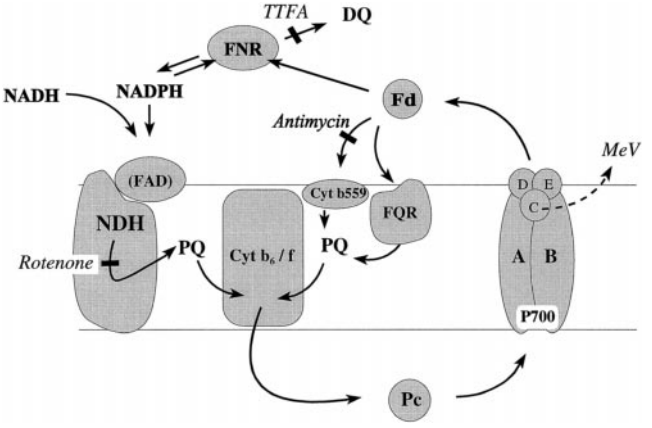

Figure 7. Model of cyclic electron transport in thylakoid membranes of barley, showing the site of action of inhibitors and artificial acceptors (italics) used in this study. A, B, C, D, and E, Subunits of PSI; DQ, duroquinone; FQR, antimycin-insensitive Fd:quinone reductase; and Pc, plastocyanin.

vated samples (Fig. 6). NDH is localized in the grana margins and stroma lamellae (Steffánsson, 1996). The mechanism of ionic activation could be related to a destacking of the thylakoid membranes and exposure of previously masked pools of NDH. Because the activities observed upon light treatment or destacking are not additive effects, it is tempting to speculate that light activation leads to a lateral displacement of the NDH complex toward the stroma lamellae. Present investigations are aimed at determining the mechanisms of activation.

A model of the different pathways of cyclic electron transport is shown in Figure 7. Fd-catalyzed cyclic electron transport may be mediated in an antimycin-sensitive reaction by a low potential Cyt b_{559} (Miyake et al., 1995). The antimycin-insensitive Fd:quinone reductase has not been identified. Possibly, the antimycin-insensitive Fd:quinone reductase and NDH are identical. Ongoing experiments with inhibitors of NDH and Fd:quinone reductase should allow us to decide between these possibilities.

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