Identification of the Ndh (NAD(P)H-Plastoquinone-oxidoreductase) Complex in Etioplast Membranes of Barley: Changes during Photomorphogenesis of Chloroplasts

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In the last few years the presence in thylakoid membranes of chloroplasts of a NAD(P)H-plastoquinone oxidoreductase complex (Ndh complex) homologous to mitochondrial complex I has been well established. Herein, we report the identification of the Ndh complex in barley etioplast membranes. Two plastid DNA-encoded polypeptides of the Ndh complex (NDH-A and NDH-F) were relatively more abundant in etioplast membranes than in thylakoids from greening chloroplasts. Conversion of etioplast into chloroplast, after light exposure of barley seedlings grown in the dark, was accompanied by a decrease in the NADH dehydrogenase activity associated to plastid membranes. Based on the mentioned observations, the plastid Ndh complex (Friedrich et al. 1995) — Etioplasts — Peroxidase — Photomorphogenesis.

Key words: Barley (Hordeum vulgare) — Etioplasts — NAD(P)H-plastoquinone oxidoreductase complex (Ndh complex) — ndh genes — Peroxidase — Photomorphogenesis.

Chloroplasts contain a NAD(P)H dehydrogenase (Ndh) complex which includes several polypeptides homologous to mitochondrial and eubacterial respiratory complex I subunits (Sazanov et al. 1998a). These polypeptides are encoded in eleven reading frames (ndhA-K) present in the plastid DNA derived from the majority of vascular plants (Sugiura 1992). Early evidences indicated that all the ndh genes were transcribed (Matsubayashi et al. 1987). Further investigations have identified the polypeptidic products of most of these genes in thylakoid membranes of higher plants (Berger et al. 1993, Guedeney et al. 1996, Martín et al. 1996, Catalá et al. 1997). Likewise, the presence of an active Ndh complex was demonstrated in thylakoids of barley (Quiles et al. 1996) and potato (Guedeney et al. 1996). More recently the purification of the Ndh complex from thylakoids of pea has been reported (Sazanov et al. 1998a). Nonetheless, many questions concerning the Ndh complex remain unanswered. In the first place, little is known about the structure of the Ndh complex. Until now the speculation concerning the structure of the Ndh complex was based solely on models previously designed for mitochondrial and bacterial complex I (Friedrich et al. 1995). The E. coli complex I (which is supposed to represent a minimal form of the NADH dehydrogenase) contains 14 subunits, in contrast with the 11 subunits encoded in the plastid genome. The three subunits that are not codified in the plastid genome would correspond to the homologues of the bacterial subunits involved in the binding and oxidation of NADH (Friedrich et al. 1995). Thus, some authors have proposed that FNR (ferredoxin NADP oxidoreductase) could transfer electrons from NADPH to the plastid Ndh complex (Friedrich et al. 1995, Guedeney et al. 1996) as an alternative electron input device. The main proof supporting this hypothesis was found in immunodetection of FNR associated with the plastid Ndh complex after detergent solubilization of thylakoids and separation of membrane proteins by non-denaturing electrophoresis in potato (Guedeney et al. 1996) or barley (Quiles and Cuello 1998). On the other hand, Burrows et al. (1998) did not found any association of FNR with the Ndh complex after detergent solubilization and blue-native-PAGE of thylakoid proteins from pea. The same group reported that the NADH dehydrogenase activity of the purified complex was much higher than the NADPH dehydrogenase activity (Sazanov et al. 1998a) and again furnished evidence which indicated that the Ndh complex was not associated to FNR. Recently the group of Peltier (Corneille et al. 1998) has also

Abbreviations: AAC, mitochondrial ADP/ATP carrier protein; FeCN, ferricyanide; FNR, ferredoxin-NADPH-oxidoreductase; LSU, large subunit of Rubisco; NBT, Nitroblue tetrazolium; Ndh, NADH-plastoquinone-oxidoreductase.

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suggested that the NAD(P)H dehydrogenase activity of the Ndh complex is independent of the NADPH dehydrogenase activity related to FNR.

Nonetheless, there is a general agreement about that plastoquinone could be the electron acceptor associated to the Ndh complex (Corneille et al. 1998, Feild et al. 1998, Sazanov et al. 1998a).

However, the most important dilemma consists on establishing the physiological role of the Ndh complex. Two main hypotheses have been proposed: the first alleges that the Ndh complex participates in photosynthetic cyclic electron transport; the second one proposes the idea of its involvement in chlororespiration, a putative chloroplastic respiratory process. The evidences favouring the first hypothesis include the finding which shows that the relative amounts of several subunits of the complex in C4 plants are substantially increased in bundle sheath plastids (which present high levels of cyclic electron transport and low levels of linear electron transport) as compared to mesophyll chloroplasts with lower levels of cyclic transport (Kubicki et al. 1996). More recent evidences obtained by a reverse genetic approach suggest that cyclic electron flow around PSI is impaired in ndhB deficient tobacco transformants (Shikanai et al. 1998). On the other hand, several studies indicate the existence of a respiratory electron transport chain in chloroplasts of green algae (Bennoun 1982, 1994) and their counterpart in higher plants (Garab et al. 1989, Lajkó et al. 1997, Feild et al. 1998). The main observations supporting chlororespiration are based on studies that indicate the capacity of chloroplasts to reduce plastoquinone in the dark and independently of cyclic electron flow. The dark reduction of plastoquinone in isolated plastids of sunflower is impaired by inhibitors of mitochondrial complex I (Feild et al. 1998). Whereas, in tobacco the inhibition is induced by mutagenesis or disruption of ndh genes (Kofer et al. 1998, Sazanov et al. 1998b). Perhaps the main drawback in accepting the chlororespiratory model derives from the lack of direct evidence concerning the existence of a terminal oxidase, which would mediate the oxidation of plastoquinone with concomitant reduction of molecular oxygen. Very recently, proof has been reported by our group that the function of this putative plastid terminal oxidase can be undertaken by a partially purified plastid membrane associated peroxidase in chloroplasts of barley (Casano et al. 2000). According to a model proposed by B. Sabater (Casano et al. 1999), plastoquinone should be reduced by NADH in a reaction mediated by the Ndh complex. Then, reduced plastoquinone should be reoxidized by electron transfer to H$_2$O$_2$ in a reaction mediated by the plastid membrane associated peroxidase. The model is completed in chloroplasts with the formation of superoxide radical by reaction of molecular oxygen with iron-reduced proteins and the subsequent dismutation of superoxide to H$_2$O$_2$ by the action of superoxide dismutase. The global result should be the consumption of two mol of NADH per mol of O$_2$ to produce 2 mol of H$_2$O.

A dual function for the plastid Ndh complex cannot be discarded. Thus, in cyanobacteria the complex homologous to the plastidial participates in both, the respiratory and photosynthetic cyclic electron transport (Mi et al. 1995). It is also possible that the plastid Ndh complex could participate in cyclic electron flow during light cycle and in a respiratory chain under dark conditions, as proposed by Burrows et al. (1998).

Most of the data obtained until now refer to the Ndh complex in chloroplasts but little is known about its presence and possible function in non-photosynthetic plastids. Recently we have described the detection of higher levels of the NDH-F polypeptide of Ndh in non-photosynthetic than in photosynthetic tissues of barley (Català et al. 1997). Fischer et al. (1997) obtained similar results for the NDH-H and NDH-K polypeptides in maize, rice and mustard. The present paper deals with the identification of an active Ndh complex in etioplast membranes. Conversion from etioplasts into chloroplasts through light irradiation of dark-grown seedlings is accompanied by reduction of the NADH dehydrogenase specific activity associated with the Ndh complex. This change is parallel to a reduction of peroxidase activity in plastid membranes. Our data are in accordance with the participation of the Ndh complex in an electron transport chain from NADH to H$_2$O$_2$ (Zapata et al. 1998, Casano et al. 2000) that could eventually be associated with the generation of a proton gradient in non-photosynthetic tissues.

**Materials and Methods**

_Growth of plants—_Seeds of barley (_Hordeum vulgare_ L. cv. Hassan) were germinated on moist vermiculite at 23°C for 6 d in total darkness to obtain etiolated plants. Unless otherwise stated, greening leaves were obtained by exposure of etiolated plants to continuous light of 20 W m$^{-2}$ at 23°C for 24 h. All manipulations of dark grown plants were performed in total darkness.

_Preparation of plant extracts and subcellular fractions—_All preparation steps were carried out at 4°C and when possible in the dark. Total protein extracts from leaves were obtained basically as described previously (Català et al. 1997) except that extraction was carried out in a buffer composed by 50 mM Tris-HCl pH 8.0, 1% SDS, 10 mM Na-EDTA, 100 mM LiCl, 0.5 mM PMSF. To obtain etioplasts, etiolated leaves were excised and immediately afterwards placed in ice-cold grinding buffer (50 mM HEPES-NaOH pH 7.6, 0.33 M sorbitol, 2 mM ascorbic acid, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 2 mM Na-EDTA, 0.1% BSA) and briefly homogenised in an Omni-mixer. The homogenate was filtered through six layers of muslin and a layer of cotton wool. The filtrate was then centrifuged at 2,000 × g for 6 min in an SS-34 rotor. The pellet was resuspended in a small volume of grinding buffer and centrifuged on 40% percoll in grinding buffer at 30,000 × g for 30 min in the SS-34 rotor. After centrifugation the lower yellow band containing intact etioplasts was recovered, diluted ten-fold with washing...
buffer (50 mM HEPES-NaOH pH 7.6, 0.33 M sorbitol, 2 mM Na$_2$-EDTA) and sedimented at 2,500 × g for 10 min. The resulting pellet was washed again to obtain intact etioplasts.

Greening chloroplasts were obtained from greening leaves in a similar way, except that the purification of intact plastids was performed on 50% percoll in grinding buffer.

Purification of the membrane fraction of etioplasts or green-ochloroplasts was achieved upon submitting purified plastids to an osmotic shock in hypotonic buffer (50 mM HEPES-NaOH pH 7.6, 4 mM MgCl$_2$, 0.3 M NaCl, 2 mM Na-EDTA, 0.5 mM PMSF). The lysed plastids were centrifuged at 10,000 × g for 10 min and the pellet was resuspended in a buffer containing 50 mM HEPES-NaOH pH 7.6, 4 mM MgCl$_2$, 0.3 M NaCl, 2 mM Na-EDTA, 0.5 mM PMSF. After 30 min of incubation on ice, plastid membranes were recovered by centrifugation at 10,000 × g for 10 min and washed twice in washing buffer.

- Mitochondria were obtained from etiolated plants basically as described by Sandalio et al. (1987), except for the grinding buffer which was similar to the one described above for etioplast purification and centrifugation in a continuous 5-50% (v/v) percoll gradient in grinding buffer.

Enzymatic assays—NAD(P)H dehydrogenase activities were determined using FeCN as electron acceptor according to the procedure described by Cuello et al. (1995) which involves continuous measurement of the decay of A$_{420}$ caused by reduction of FeCN.

NADH-cytochrome c reductase activity was measured according to Schweitzguebel and Siegenthaler (1984), following the change in A$_{450}$ caused by the NADH dependent reduction of cytochrome c. The reaction mixture contained 0.05 mM cytochrome c, 1 mM KCN, 0.2 mM NADH and 10 μl of protein extract (including 10-40 μg of protein) in 10 mM phosphate buffer pH 7.2 and a final volume of 1 ml. The reaction was started by addition of NADH.

4-methoxy-a-naphthol and hydroquinone dependent peroxidase activities were measured according to Zapata et al. (1998) and Ferrer et al. (1990) respectively.

In all cases one unit (U) of enzyme activity was defined as such that transforms 1 μmol of substrate (NADH in the case of NADH-FeCN reductase activity) min$^{-1}$. Specific activities are referred to mg of protein.

Gel electrophoresis and zymograms—SDS-PAGE was carried out as described by O’Farrel (1975). Tricine-SDS-PAGE was performed in 10% total acrylamide gels according to the method described by Schägger and von Jagow (1987). Native-PAGE was performed at 4°C in 7% total acrylamide gels as described previously (Quiles and Cuello 1998) after solubilization of samples with 2% Triton X-100. Blue-native-PAGE was carried out in a linear gradient of 6-12% of polyacrylamide as described by Schägger et al. (1994) after solubilization of samples with 1.5% n-dodecyl-β-D-maltoside.

NAD(P)H dehydrogenase activities were detected by pyridine nucleotide dependent reduction of nitroblue tetrazolium (NBT) on gels after native-PAGE as described by Cuello et al. (1995).

Methoxinaphthol dependent peroxidase activities were detected on gels after native-PAGE as described by Ferrer et al. (1990).

Immunoblot analysis—After SDS-PAGE or Tricine-SDS-PAGE, polypeptides were transferred from gels to PVDF membranes (Immobilon-P, Millipore) as described by Towbin et al. (1979). After incubation with primary antibody, immunodetection was performed using goat anti-rabbit Ig-G antiserum linked to horseradish peroxidase (Bio-Rad) as the secondary antibody. Color was developed using tetramethyl-bencidine and H$_2$O$_2$ as substrates.

The anti-NDH-F antibody is the same as that described in Catalá et al. (1997). Anti LSU and anti-NDH-A antibodies were described in Martín et al. (1996). The anti-AAC antibody is a gift of Dr. C. Klanner (Institut für Physiologische Chemie, Ludwig Maximilians University of Munich, Germany). Dr. J. Gualberto (IBMP du CNRS, Strasbourg, France) kindly donated the anti NAD-9 antibody and Dr. Alice Barkan (University of Oregon, U.S.A.) provided the anti-D2 antibody.

Other procedures—Protein was determined, after 2% SDS solubilization of samples, with a Bio-Rad detergent-compatible protein assay kit based on the method of Lowry et al. (1951). Chlorophyll and carotenoids were determined according to Lichtenthaler (1987). Semiquantitative analysis of NDH-F was performed by densitometry of immunolabelled membranes using an UVP Easy digital image analyzer.

Results

Changes in the levels of NDH polypeptides during leaf greening—One of the most important changes occurring during greening or de-etiolation of dark grown seedlings after their exposure to light is the conversion of etioplasts into chloroplasts. This conversion implies the transformation of the etioplast membranes into thylakoids, which is concomitant with the light-mediated synthesis of chlorophyll from protochlorophyllide and the accumulation of several light-induced thylakoid proteins as the D1 and D2 proteins of PSII or the apoproteins of the light-harvesting complexes (Kirk and Tilney-Bassett 1978, Klein and Mullet 1987). According to this model, when the barley seedlings, grown for 6 d in complete darkness, were exposed to continuous light of low intensity (20 W m$^{-2}$) a rapid accumulation of chlorophyll was observed concomitantly with slower accumulation of carotenoids from whole leaf extracts. This accumulation of pigments was continuous until 48 h of exposure to light, and reached to a maximum (Fig. 1A). Similarly the D2 protein, which was not visualized by immunodetection after western blotting of whole leaf extracts from etiolated plants, eventually accumulated after 24 h of exposure to light (Fig. 1B, anti-D2). As described previously (Klein and Mullet 1987) we found out, using an anti-ATP-A antibody, that the α subunit of the plastid ATPase was already present in etiolated leaves and its relative amount with respect to a total protein basis remained constant during greening (Fig. 1B, anti ATP-A).

At least two subunits (NDH-A and NDH-F) of the plastid Ndh complex were also present in etiolated leaves, but in contrast with the behaviour of the D2 protein or the α subunit of ATPase, a decrease in the levels of the NDH-A and NDH-F polypeptides after 24 h of light exposure was observed (Fig. 1B, anti NDH-A and anti NDH-F, respectively). This decrease was similar to that found in NAD-9 subunit of the mitochondrial NADH dehydrogenase complex (Fig. 1B, anti-NAD-9) using a polyclonal antibody prepared against a wheat NAD9 fusion protein (Lamattina Maximilians University of Munich, Germany). Dr. J. Gualberto (IBMP du CNRS, Strasbourg, France) kindly donated the anti NAD-9 antibody and Dr. Alice Barkan (University of Oregon, U.S.A.) provided the anti-D2 antibody.

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et al. 1993). When the variation in the levels of NDH-F during greening was examined more meticulously, a rapid decrease in the relative amount of this polypeptide when compared, as above, with the total amount of protein from whole leaf protein extracts was also found. In fact, levels of NDH-F fell to 40% of those found in etiolated leaves after only 6 h to light exposure, and then dropped more gradually down to 20% of the original level, when plants were exposed to light for a total of 72 h (Fig. 1A).

Isolation and characterisation of etioplasts—Although we had reported previously that the antibodies against the NDH-A (Martín et al. 1996) and NDH-F (Catalá et al. 1997) polypeptides were specific in the recognition of plastid proteins, we found it convenient to check out the results presented in the previous section with purified etioplasts. It is specially critical to remove any possible cross-reaction of the anti-NDH-A and anti-NDH-F antibodies with mitochondria. It is also important to remove mitochondrial contamination in our etioplast preparations for the sake of further experiments.

Etioplasts and mitochondria were isolated from 6d barley seedlings grown in complete darkness as described in Materials and Methods. As shown in Fig. 2A, the polypeptide pattern of etioplast by SDS-PAGE was different from the polypeptide pattern of mitochondria isolated from the same starting plant material. Two main polypeptides of about 55 and 37 kDa present in the etioplast fraction (which must correspond to LSU and NADPH protoclorophyllide oxidoreductase, respectively) were barely detected in the mitochondrial fraction. Otherwise, polypeptides of around 34, 30, 25, 19 kDa and several high molecular weight polypeptides from mitochondrial fraction had not any counterpart in the etioplast fraction. Furthermore, as shown in Fig. 2B, immunoblotting assays with the anti-AAC and anti-NAD-9 antibodies directed against mitochondrial proteins showed that these antibodies recognised specifically their respective antigen in mitochondrial but not in etioplast preparations. Thus, it was concluded that our etioplast preparations were free of mitochondrial contamination. On the other hand, the etioplast fraction was recognised by antibodies prepared against plastid proteins as LSU. The same antibodies did not react with polypeptides of the mitochondrial fraction.

It is significant that the anti NDH-F antibody, which recognised a polypeptide from etioplast fraction, did not react with the mitochondrial fraction (Fig. 2B). In a similar way, the anti-NDH-A antibody recognised specifically plastid polypeptides, but not mitochondrial ones (not shown). These results are not surprising as far as the anti NDH-A and anti NDH-F antibodies were raised against specific antigenic sites present in the plastid corresponding polypeptides (Martín et al. 1996, Catalá et al. 1997). Additionally, when cytochrome c reductase activity was measured as a marker of mitochondria it was found to exceed about 18-fold in mitochondria (0.086 U mg\(^{-1}\)) as compared with etioplast preparations (0.005 U mg\(^{-1}\)). Considered as a whole, these results clearly showed that: (1) our etioplast preparations were essentially free of mitochondrial contamination; (2) the anti-ndh-F antibody reacted with etioplast but not with mitochondria. On the
other hand, detectable levels of Rubisco in etioplasts of barley and other plants has been reported previously (Klein and Mullet 1987, El Amrani et al. 1994). Thus, the presence of Rubisco, a high carotenoid content and the absence of chlorophyll indicated the effective presence of etioplasts in our preparations.
Changes in plastid membranes during greening—It has been reported (Klein and Mullet 1987) that the main differences between barley etioplasts and greening chloroplasts (chloroplasts developed from etioplasts after exposure of the plants to light) are related to the membrane fraction of plastids. When we compared the polypeptide composition of etioplast membranes and thylakoid preparations, such differences were rather evident (Fig. 3). A main polypeptide of 37 kDa, corresponding to NADPH-protochlorophyllide-oxidoreductase (Reinbothe et al. 1996), present in etioplast membranes, almost disappeared in thylakoids isolated from etiolated plants exposed to 24 h of continuous light. Otherwise, several polypeptides of 28–23 kDa (putatively the LHCII apoproteins) absent in etioplast membranes represented majority in thylakoids of the de-etiolated plants (Fig. 3A). As found in total protein extracts from leaves, the immunodetected a subunit of ATPase was present in similar levels in etioplast membranes and thylakoids and the D2 protein of PSII was only detected in thylakoids (Fig. 3B). As stated above, all these results were consistent with those described for barley during the transformation from etioplast to chloroplasts (Klein and Mullet 1987). In contrast to the accumulation of D2 protein in plastid membranes during greening, we have found a decrease of the NDH-F and NDH-A polypeptides that was consistent with the results obtained in whole leaf protein extracts.

NADH and NADPH dehydrogenase activities decreased in plastid membranes during greening—The NDH-A and NDH-F polypeptides are components of a partially characterized thylakoid NADH dehydrogenase complex (Martin et al. 1996, Catalá et al. 1997). Nonetheless, the results described until now give rise to the following questions: is there an active Ndh complex similar to that described in thylakoid membranes already present in etioplasts? Could this complex be more active in etioplasts than in chloroplasts of young leaves? To answer these questions we have first measured the total NADH and NADPH dehydrogenase activity in soluble and membrane fractions of etioplasts and greening chloroplasts using FeCN as electron acceptor. Results listed in Table 1 prove that there were significant changes in both NADH and NADPH activities during greening. The main changes were observed upon comparison of the NADH or NADPH activities relative to etioplast membranes with those in thylakoid of greening chloroplasts. Thus, a decrease of about 90% for NADH dehydrogenase activity and around 60% in the NADPH dehydrogenase activity were measured for plastid membrane fractions during greening. However, when the activities in the soluble fractions of plastids were compared, only a 20% decrease in the NADH activity was found, while the levels of NADPH activity remained constant after the 24 h continuous light treatment. Most of the decay in NADPH activity associated with the membrane fraction could be related to the well-characterised (Reinbothe et al. 1996) decrease in NADPH-protochlorophyllide oxidoreductase activity during greening. These data were also well correlated with the observed decrease of the 37 kDa polypeptide present in our etioplast membrane preparations. Otherwise, the NADPH dehydrogenase present in the soluble fractions of etioplasts and greening chloroplasts might be partially related to FNR activity that is present in non-photosynthetic plastids such as leucoplasts (Morigasaki et al. 1990), chromoplasts (Green et al. 1991) and probably etioplasts (Scheumann et al. 1998). Finally, changes measured in membrane-associated NADH dehydrogenase activity could be tentatively attributed to the observed decrease of the relative amounts of the NDH-A and NDH-F polypeptides during greening.

An active NADH dehydrogenase complex is associated to etioplast membranes—To assess the feasibility of the latter, we carried out zymograms to detect the different membrane associated NAD(P)H dehydrogenase activities in etioplasts and greening chloroplasts after separation by native gel electrophoresis of membrane proteins solubilized with Triton X-100. Obtained results revealed the presence of five major bands with NADH dehydrogenase activity (d1–d5) and a larger number of NADPH dehydrogenase activities in etioplast membranes (Fig. 4). After 24 h of light exposure, the d1 and d3 bands with NADH activity

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<th>Substrate</th>
<th>Fe-CN NAD(P)H dehydrogenase activity (μmol NAD(P)H oxidized min⁻¹ (mg protein)⁻¹)</th>
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<td>Etioplast Membranes</td>
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<tr>
<td>NADH</td>
<td>0.43 ± 0.08 0.63 ± 0.15</td>
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<tr>
<td>NADPH</td>
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Data are the means ± SD of at least 3 different experiments.
Fig. 4 Changes in NADH and NADPH dehydrogenase plastid membrane activities during greening. Etioplast membranes (0) and thylakoids isolated from greening chloroplasts of plants grown for 6 d in the dark and for 24 additional hours in continuous light (24) were electrophoresed under native conditions and NADH or NADPH activities detected on gels as described in Materials and Methods. 100 μg of total protein were loaded per lane.

were missing in the thylakoids of the resulting greening chloroplasts and only the d2 NADH dehydrogenase activity band was clearly detected. It is noteworthy that less pronounced changes were observed upon comparison of NADPH activities relative to etioplasts and greening chloroplasts. Activities associated with d1 and d3 bands were apparently NADH but not NADPH dependent. In principle, activity associated with d5 band seemed to be NADPH dependent and activity associated with d2 band was probably both NADH and NADPH dependent. Although there was an NADPH dependent band with a migration similar to the NADH-dependent d4 band, the two activities might correspond to two different proteins as far as d4 activity apparently decrease during greening meanwhile NADPH activity increase during greening.

The bands with NADH dehydrogenase activity from etioplast membranes detected by native-PAGE were excised and ran in a SDS-PAGE second dimension followed by a subsequent western blotting and immunodetection with the anti-NDH-F antibody. This antibody recognised clearly the d1 band and only barely the d2 band (Fig. 5). These results indicated that the NDH-F polypeptide was associated with d1 band. The faint signal associated with d2 band was probably due to the presence of a small amount of Ndh polypeptides contaminating this band which also contained small amounts of other plastid components like Rubisco (not shown). Similar results were obtained when anti-NDH-A antibody was employed (not shown). Thus, very probably, the d1 band with NADH-dependent dehydrogenase activity contained the complex that included the ndh plastid-encoded polypeptides. Silver-staining after Tricine-SDS-PAGE of the d1 band from etioplast membranes revealed that it contained polypeptides of 75, 70, 55, 40, 34, 28, 26, 24, 22, 19 and 14 kDa (Fig. 5). After immunodetection, the 70 kD polypeptide was found to correspond to the NDH-F polypeptide (Fig. 5). The molecular weights of most of the other polypeptides detected were consistent with those described for the NADH-dehydrogenase complex purified from pea (Sazanov et al. 1998a) or barley (Quiles et al. 1996) thylakoids.

In addition, the d1 band was ran in a second dimension by blue-native-PAGE. Blue-native-PAGE separates oligomeric proteins by size (Schägger et al. 1994) and consequently, it is currently used to determine the overall molecular weight of protein complexes. As shown in Fig. 6 (lane d1), after second dimension in blue-native-PAGE of the d1 excised band, a main band of approximate molecular weight of 580 kDa and a secondary band with roughly 500 kDa were observed. Noteworthy, Burrows et al. (1998) described that after partial solubilization of tobacco chloroplasts with n-dodecyl-maltoside two protein complexes of 550 and 200 kDa, both containing Ndh polypeptides, were separated by blue-native-PAGE. These authors sus-
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Figure 6 Separation of etioplast membrane complexes by blue-native-PAGE. Etioplast membranes (em) were solubilized with n-dodecyl-maltoside and run in blue-native-PAGE as described in Materials and Methods. The d1 band with NADH-dehydrogenase activity excised from a native gel was run in a parallel lane as indicated in Fig. 5. Chloroplast (Chi) from 7 d old plants were run in a third lane for comparison and detection of Rubisco. On the left are indicated the migration of Rubisco (540 kDa), F1 ATPase subcomplex (470 kDa), myosin (200 kDa) and β-galactosidase (116 kDa).

Figure 7 Identification of the NADH dehydrogenase complex in etioplast membranes after blue-native-PAGE. n-dodecyl-maltoside solubilized etioplast membranes were separated by blue-native-PAGE in the first dimension followed by denaturing Tricine-SDS-PAGE in the second dimension and detection with antibodies specific for NDH-A and NDH-F. For comparison, immunodetection of plastid ATPase was also included.

It was found that the 550 kDa band corresponded to the whole Ndh complex meanwhile the 200 kDa corresponded to a subcomplex formed during the isolation procedure (Burrows et al. 1998). In our case, the larger band of 580 kDa could correspond to the 550 kDa Ndh complex described by Burrows et al. (1998), while the secondary 500 kDa band may be the consequence of formation of a Ndh subcomplex by detergent solubilization or the presence in d1 band of other membrane components not related with the Ndh complex. As shown in Fig. 6 (em lane), several large size complexes from etioplast membranes can be resolved by this method. One of these complexes comigrated with the 580 kDa band of the d1 NADH activity. This band represented about 5% of the total protein content of etioplast membranes. The lane containing the etioplast membrane protein complexes resolved by blue-native-PAGE was excised and run on a second dimension Tricine-SDS-PAGE. After western blotting of the gel, immunodetection with the anti-ndh-A and anti-ndh-F antibodies showed that the 580 kDa etioplast membrane protein complex, which comigrated with the 580 kDa d1 blue-native-resolved band, contained the NDH-A and NDH-F polypeptides of the Ndh complex (Fig. 7). For comparison, Fig. 7 shows anti-ATP-A detection of the plastid ATPase F1 subunit which corresponded to the 470 kDa main band, previously detected by blue-native PAGE.

Changes in etioplast membrane-associated peroxidase activity are parallel to changes in NADH-dehydrogenase activity during greening—The above reported experiments, established the presence of an active NADH-dehydrogenase complex containing products of the plastid ndh hydrogenase genes associated with etioplast membranes. Assuming that most evidences reported until now indicate that the activity of this complex reduces plastoquinone, the following question comes to mind: which component of plastids would reoxidize reduced plastoquinone? It has been proposed recently (Zapata et al. 1998) that a thylakoid-hydroquinone-peroxidase showing in vitro activity on reduced plastoquinone is also capable to reoxidize reduced plastoquinone in vivo. This peroxidase constitutes an adequate alternative to a reluctant terminal oxidase activity for a putative electron transport chain associated with the NADH dehydrogenase complex in plastid membranes. More recent works of our group show that the activity of purified Ndh dehydrogenase (which is inhibited by the anti-NDH-A antibody) can be coupled to the activity of a semipurified thylakoid hydroquinone peroxidase in vitro (Casano et al. 2000). To test these previous results we have measured the peroxidase activity associated with purified etioplasts or greening chloroplasts membranes. It was found that changes in peroxidase activity (Table 2) are compatible with the model proposed by B. Sabater (Casano et al. 2000) when measured using two different substrates (hydroquinone and 4-methoxy-a-naphthol). Peroxidase activity was detected in plastid membranes, but not in the soluble fraction of plastids. This activity decreased by about 70% during greening. The reduction in specific activity of the plastid membrane-associated-peroxidase during greening is comparable to the decrease of the NADH dehydrogenase activity associated with the plastid membranes. The barley plastid membrane peroxidase activity was identified by native-PAGE electrophoresis as a low migrating band (Fig. 8) prominent in etioplast membranes.
Fig. 8 Identification of peroxidase activities in plastid membranes. After native-PAGE, peroxidase activities associated with etioplast membranes (0) or thylakoids (24) were detected as described in Materials and Methods. Etioplast membranes and thylakoids were isolated, respectively, from 6 d plants grown in total darkness or plants grown under the same conditions and exposed to continuous light for additional 24 h. Arrow indicates the migration of the peroxidase activity detected in etioplast membranes. The position of pigmented bands corresponding to chlorophyll complexes in thylakoids is also indicated.

but barely detectable in thylakoid membranes of greening chloroplasts. Migration of the peroxidase activity associated with etioplast membranes in native-gels is similar to that described for the thylakoid-associated peroxidase in mature chloroplasts (Zapata et al. 1998).

Discussion

Most of the work carried out until now on the Ndh complex has been performed in chloroplasts. Thus, little is known about the presence and possible function of the Ndh complex in non-photosynthetic plastids as etioplasts. Here we supply evidence which proves that the Ndh complex is present in etiolated leaves of barley. In fact, we have found that the levels of the NDH-A and NDH-F polypeptides, when related to the total protein of barley leaves, diminished during greening. This is in clear contrast with the accumulation of photosynthetic pigments and PSII proteins after exposure to light of dark grown seedlings (Fig. 1). Although a similar behaviour was found for the NAD-9 polypeptide of the mitochondrial complex I, it is unlikely that our results are due to unspecific reactions of the anti-NDH-A and anti-NDH-F antibodies with mitochondria (Fig. 2). Furthermore, similar results were obtained when comparing highly purified membrane preparations, to which are associated the NDH-A and NDH-F polypeptides, from etioplasts and greening chloroplasts (Fig. 3). Consistent with the presence of a functional Ndh complex in etioplast membranes and based on the data discussed above, it was found that the NADH dehydrogenase and NADPH dehydrogenase specific activities associated with etioplast membranes were higher than those associated with thylakoid membranes of greening chloroplasts obtained after 24 h of light exposure (Table 2). Interestingly, such decrease was higher for NADH than for NADPH specific activity. When the different NAD(P)H dehydrogenase activities associated to etioplast membranes were resolved by native-PAGE, it was found that the NDH-F and NDH-A polypeptides were present in the low-migrating band d1 which showed NADH but not NADPH dehydrogenase activity (Fig. 4, 5). This behaviour is similar to the one described for the partially purified Ndh complex of pea (Sazanov et al. 1998a) and indicate that, at least in the case of barley etioplasts, is improbable that the use of NADH as substrate of the Ndh complex could be related to FNR. According to Fig. 6 and 7, it seems feasible that the NADH dehydrogenase activity associated with the NDH-A and NDH-F polypeptides forms a large protein complex in etioplast membranes. The overall molecular weight of this complex is about 580 kDa which agrees with the molecular weight previously reported for the pea chloroplast Ndh complex (Sazanov et al. 1998a). It can be argued that the measured decrease in total membrane associated NADH dehydrogenase activity during greening is less than 100% (in fact about 90%), while the d1 band almost disappeared under the same conditions. However, these results most likely indicate that not all the NADH

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Peroxidase activity</th>
<th>Etioplast</th>
<th>Greening chloroplast</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(µmol substrate oxidized min⁻¹ (mg protein)⁻¹)</td>
<td>Soluble Membranes</td>
<td>Soluble Membranes</td>
</tr>
<tr>
<td>Methoxinaphthol</td>
<td>0</td>
<td>0.060±0.006</td>
<td>0</td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>0</td>
<td>0.595±0.085</td>
<td>0</td>
</tr>
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Data are the means±SD of at least 3 different experiments.
dehydrogenase activity associated to plastid membranes is related with the Ndh complex, as can be observed in Fig. 4. From the data reported by Sazanov et al. (1998a) it can be deduced that less than 30% from the total NADH dehydrogenase activity associated with thylakoids of mature tobacco chloroplasts is due to the Ndh complex activity. Furthermore, the detection of low levels (when compared with etioplasts) of the NDH-A and NDH-F polypeptides in greening chloroplasts (around 20% of level present in etioplasts) does not necessarily mean that the complex is fully active in these plastids. Anyhow, our results show the presence of an active Ndh complex in etioplasts and a clear decrease of its relative amount and specific activity in plastid membranes during greening.

The presence of the Ndh complex in etioplasts may be relevant in view of doubts that concern its function in plastids and the biogenesis of chloroplasts. According to our results light is not required for the expression of ndh genes during chloroplast biogenesis. However, the observed decrease of the Ndh complex relative levels in plastid membranes during greening might be explained more precisely by massive accumulation of photosynthesis related proteins, rather than by degradation of the ndh genes products. Significant levels of Ndh polypeptides have been found in other non-photosynthetic tissues of barley, including the leaf basal meristem of barley (Catala et al. 1997), which suggest that synthesis of the plastid Ndh complex may be an early event during the differentiation of proplastids to other kinds of plastids.

In thylakoids of pea the Ndh complex is a minor fraction (about 0.2%) of membrane protein (Sazanov et al. 1998a). Although we have found higher levels of the Ndh complex in etioplast membranes than in thylakoids of barley, the amount of the complex is still low (around 5% of total etioplast membrane protein). This does not necessarily mean that the role of the Ndh complex would be negligible, as far as ndh genes are present in the genomes of most vascular plants. In fact, as indicated above, the specific activities of NADH dehydrogenase and peroxidase are high in etioplast membranes and they are strongly diminished during greening (Table 1, 2). It probably means that its function is relevant only under some physiological conditions of the plant. As such, it can contribute to clarification of controversial reports about the viability of plastids and the biogenesis of chloroplasts. Recently Casano et al. 2000 have suggested that the Ndh complex and a thylakoidal peroxidase are involved in chlororespiration. The participation of a membrane associated plastid peroxidase in chlororespiration could account for the terminal electron acceptor that mediates the re-oxidation of plastquinone after reduction by NADH. In this sense, the parallel evolution of membrane peroxidase activity and NADH dehydrogenase activity associated with the Ndh complex during greening, supports the existence of an electron transport from NADH to H2O2 in plastids. H2O2 could be generated by superoxide dismutase (Casano et al. 2000) and by several plastid oxidase activities present in etioplasts or other non-photosynthetic plastids (Lermon-tova et al. 1997, Uchida et al. 1997, Kruse et al. 1995, Murata et al. 1997).

Thanks are given to Drs. A. Barkan and R. Maier by the anti-D2 and anti-ATP-A antibodies, to Dr. J. Gualberto by the anti NAD-9 antibody, to Dr. C. Klenner by the anti-AAC antibod- and to Dr. M. Martin by critical discussion. This work was supported by a grant from the spanish DGICYT (PB96-0675).

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(Rceived June 21, 1999; Accepted October 27, 1999)