The Expression of Subunits of the Mitochondrial Complex I-Homologous NAD(P)H-Plastoquinone-Oxidoreductase during Plastid Development

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Plastids contain a NAD(P)H-plastoquinone-oxidoreductase which is homologous to the eubacterial and mitochondrial NADH-ubiquinone-oxidoreductase (complex I), but the metabolic function of the enzyme is still not yet understood. The enzyme consists of at least eleven subunits (NDH-A-K), which are all encoded in the plastid chromosome. In this study we have investigated the tissue-specific and light-dependent expression of the subunits NDH-H and NDH-K in maize, rice and mustard by western blot analysis. No NDH-proteins were found in root tissue, indicating that the presence of the enzyme is confined to leaf plastids. Analysis of the expression during the light-dependent development from etioplasts to chloroplasts showed that high amounts of NDH-H and -K are present in etioplasts. The same result was found for subunits of the ATPase. In contrast, components of the photosynthetic electron transport chain (PSII-B, cytochrome f and PSI-D) accumulated only after illumination. In an second investigation, the expression of NDH-proteins along the natural chloroplast developmental gradient from proplastids to chloroplasts in light-grown maize leaves was analysed. NDH-H and NDH-K as well as the ATPase were present at the youngest stages of chloroplast development, while the massive accumulation of subunits of the photosystems and the cytochrome b_6/f-complex took place in older leaf sections. We conclude from these studies that a functional NAD(P)H-plastoquinone-oxidoreductase is present in etioplasts and developing plastids. We suggest that the enzyme serves the generation of a proton gradient across the prothylakoid membrane that is necessary for protein integration into the membrane at developmental stages where a functional photosynthetic electron transport chain is not yet operating.

Introduction

Cyanobacteria and chloroplasts of higher plants harbor an NAD(P)H-plastoquinone-oxidoreductase that is homologous to the NADH-ubiquinone-oxidoreductase (complex I) of bacteria and mitochondria (Friedrich *et al.*, 1995). This enzyme is composed of at least eleven subunits (NDH-A to K), which are all encoded in the plastid chromosome (Sugiura, 1992). It has been shown that the enzyme is located on the stromal thylakoid system (Berger *et al.*, 1993).

In bacteria and mitochondria, complex I oxidizes NADH and forms the first proton-pumping complex of the respiratory chain (Friedrich *et al.*, 1995), however the function of the homologous plastidial enzyme has not yet been clarified. So far, three possible functions have been proposed. Firstly, the enzyme may be part of a so called chlororespiratory pathway which serves the oxidation of surplus reduction equivalents generated via the oxidative pentose phosphate pathway during starch degradation in the dark (Bennoun, 1982). Secondly, the enzyme may be a component of the cyclic electron transport pathway around photosystem I. Support for this hypothesis comes from the finding that in the bundle sheath plastids of C_4-plants, which rely on cyclic electron transport to fullfil their ATP requirements for CO_2 fixation, the relative amounts of NDH-proteins are increased as compared to mesophyll plastids (Kubicki *et al.*, 1996). Thirdly, the enzyme may be involved in the detoxification of deleterious oxygen species in senescent chloroplasts or under conditions of photooxidative stress (Martin *et al.*, 1996).

For a better understanding of the function of the NAD(P)H-plastoquinone-oxidoreductase in plastid energy metabolism, we have investigated, whether the enzyme is expressed in other plant organs, i.e. roots, and studied the expression of the

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enzyme during light-dependent chloroplast development.

Materials and Methods

The plants were obtained from the following sources: maize (*Zea mays*, cv. B73 from Kleinwanzlebener Saatzucht, Einbeck, FRG), rice (*Oryza sativa*, cv. Lido from Societe Italiana Sementi, Italy) and mustard (*Sinapis alba* from Carl Sperling & Co., Lüneburg, FRG).

For the analysis of the light-dependent expression of NDH-proteins, maize and rice were grown at 26 °C in darknes for 7 or 8 days, respectively. Mustard was grown at 21 °C for 4 days. The plants were then illuminated for up to 24 hours with white fluorescent lamps. For the analysis of the expression along the developmental gradient from proplastids to chloroplasts in monocotyledous plants, maize was grown under greenhouse conditions for 12 days with additional illumination (14 h per day), provided by a combination of sodium and mercury high-pressure vapour lamps. At harvest, the first and second leaves were discarded and the third leaf with the enclosed younger leaves was cut into six sections (0–1, 1–2, 2–4, 4–6, 6–8 and 8-rest cm) starting from the the first nodium as described in Martineau and Taylor (1985).

Intact plastids were prepared by the homogenization of leaf material in medium A as described in Herrmann (1982) in a Waring blendor. The homogenate was filtered trough 80 μm nylon gaze and three layers of miracloth. The plastids were collected by centrifugation (2 min, 6000 x *g*), resuspended in a small volume of medium A and then layered upon a percoll density step gradient (40/85%) as described in Bartlett *et al.* (1982). Broken and intact plastids were separated by centrifugation for 10 min at 10000 x *g* in the Sorvall HB-4 rotor (Sorvall Dupont de Nemours, Bad Homburg, FRG). The band of intact plastids at the 40/85% percoll interphase was recovered from the gradient, washed two times in 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes)/KOH, pH 7.8, 330 mM sorbitol and then stored at −20 °C.

The plastids were solubilized in Laemmli's final sample buffer and separated by electrophoresis trough a 12.5% polyacrylamide gel (Laemmli, 1970). The proteins were then transferred to polyvinyidene difluoride (PVDF, Immobilon P, Millipore, Eschborn, FRG) membranes according to Kyhse-Anderson (1984). The membranes were incubated with the different antisera listed below and antigen-antibody complexes were detected with the ECL Western-blotting analysis system from Amersham Buchler (Braunschweig, FRG). The sources of the antibodies were: tobacco NDH-H and rice NDH-K (Kubicki *et al.*, 1996) and spinach PSII-B (Oswald *et al.*, 1990). The antibodies against spinach ATPase, PSI-D and cytochrome f were gifts from H. Strotmann (Heinrich-Heine-University, Düsseldorf, FRG), R. Nechushtai (Hebrew University, Jerusalem, Israel) and N. Nelson (Roche Institute, Nutley, N. J., USA), respectively.

Protein concentrations were determined according to Bensadoun and Weinstein (1976).

Results

The antisera against NDH-H and NDH-K were obtained against fusion proteins expressed in *E. coli*, which contained only parts of the reading frames of *ndhH* from tobacco and *ndhK* from rice (Kubicki *et al.*, 1996). Initially, the antisera were tested against total membrane preparations from different mono- and dicotyledonous plant species. Specific signals of the expected molecular masses were obtained with the NDH-H antibody for maize, rice and mustard and with the NDH-K antibody for maize and rice. These plants were therefore chosen for further analysis.

Since the expression of NDH-proteins has been studied so far only in chloroplasts, we analysed, if NDH-H and NDH-K are also present in roots. After western analysis of root plastids from maize and rice no specific signals were detectable for both subunits (data not shown). Thus the expression of NDH-proteins is restricted to leaves.

We then studied the expression of NDH-H and -K in leaves during the light-dependent conversion of etioplasts to chloroplasts. To allow a comparison of the expression of NDH-proteins to other components of the photosynthetic electron transport chain, the concentrations of subunits of photosystem I (PSI-D) and II (PSII-B), the cytochrome b_6/f complex (cytochrome f) and the ATPase (α- and β-subunit) were monitored in parallel. The kinetics of protein accumulation after

the illumination of dark grown maize, rice and mustard plants, respectively, are shown in Figs 1, 2 and 3. In all three plant species tested, NDH-H and NDH-K were found in etioplasts. In maize and rice, the concentrations of both NDH-proteins did not change significantly after illumination (Figs. 1 and 2), while in mustard, the relative amount of NDH-H increased (Fig. 3). A similar result was obtained for the α- and β-subunits of the ATPase. In contrast to the subunits of the NAD(P)H-plastoquinone-oxidoreductase and the ATPase, subunit D of photosystem I and subunit B of photosystem II were not detectable in etioplasts. For cytochrome *f*, different kinetics of accumulation were found in the three plant species. While in mustard cytochrome *f* was detectable only after 16 hours of illumination (Fig. 3), maize and rice etioplasts contained cytochrome *f*. A similar difference between mono- and dicotyledonous plant species in the accumulation of cytochrome *f* and other subunits of the cytochrome b_6/f complex has been reported for wheat and peas by Takabe *et al.* (1989).

A gradient of plastid development from proplastids to chloroplasts is formed in leaves of monocotyledonous plants grown under a natural day/night regime. Originating from the basal meristem, the plastids differentiate via several typical developmental stages, i.e. proplastids, amyloplasts, amoeboid plastids and prochloroplasts to chloro-

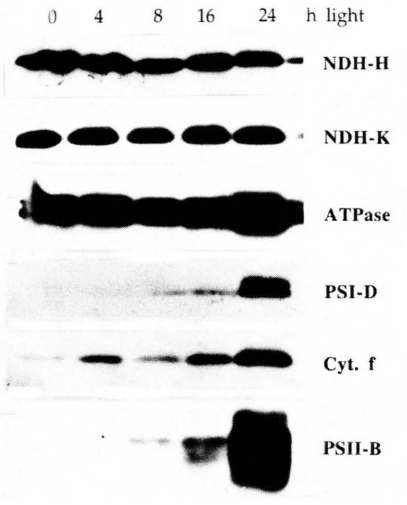

Fig. 1. Western blot analysis of the expression of NDH-H and NDH-K, the α- and β- subunits of the ATPase, and components of the photosynthetic electron transport chain (PSI-D, Cyt. *f*, PSII-B) during the greening of etiolated 7 day old maize leaves. The plants were illuminated for 4, 8, 16 to 24 hours. Each lane contains 50 μg protein. The α- and β-subunits of the ATPase run together in one band.

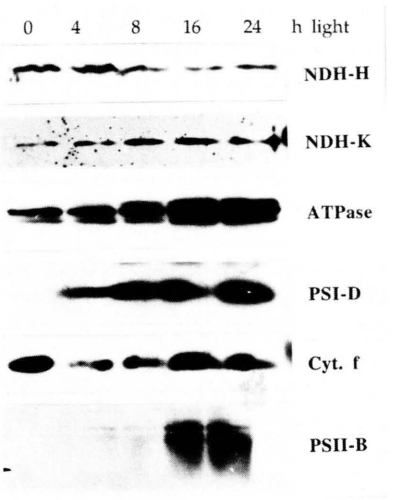

Fig. 2. Western blot analysis of the expression of NDH-H and NDH-K, the α- and β- subunits of the ATPase, and components of the photosynthetic electron transport chain (PSI-D, Cyt. *f*, PSII-B) during the greening of etiolated 8 day old rice plants. The plants were illuminated for 4, 8, 16 to 24 hours. Each lane contains 50 μg protein. The two weak bands underlying the PSII-B signal are from a preceeding incubation with the ATPase antibody.

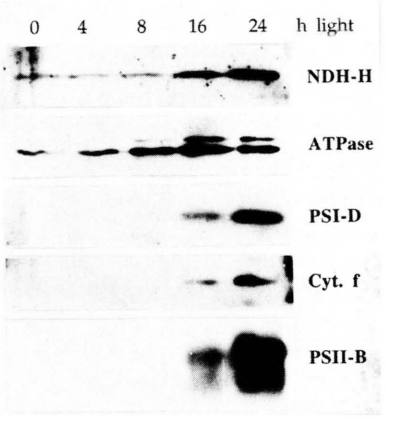

Fig. 3. Western blot analysis of the expression of NDH-H, the α- and β- subunits of the ATPase, and components of the photosynthetic electron transport chain (PSI-D, Cyt. *f*, PSII-B) during the greening of 4 day old mustard plants. The plants were illuminated for 4, 8, 16 to 24 hours. Each lane contains 50 μg protein.

plasts (Whatley, 1977; Leech, 1984). To analyse, if the expression of the NAD(P)H-plastoquinone-oxidoreductase is coupled to this development, plastids of different sections of the third leaf of a 12 day old maize plant were isolated and then tested by immunoblot analysis. NDH-H as well as the α- and β-subunits of the ATPase were found in the second leaf section only 1 cm apart from the basal meristem (Fig. 4). Their concentrations increased up to the fourth leaf section and then

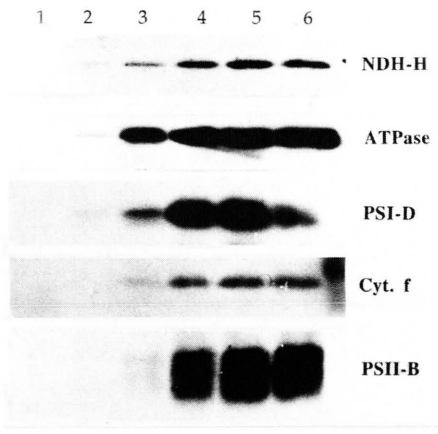

Fig. 4. Western blot analysis of the accumulation of NDH-H, the α- and β- subunits of the ATPase and components of the photosynthetic electron transport chain (PSI-D, Cyt. *f*, PSII-B) within sections of the third leaf of a 12 day old maize plant grown under a day/night regime. The sections 1–6 were: 0–1, 1–2, 2–4, 4–6, 6–8 and 8-rest cm. Each lane contains 50 μg of etio-chloroplast protein.

remained constant. PSI-D was also detectable in the second leaf section, but the main accumulation of this protein took place in sections 4 and 5 where the concentrations of the NAD(P)H-plastoquinone-oxidoreductase and the ATPase had already reached their maximal level. Low amounts of cytochrome *f* and PSII-B were detectable in the third leaf section, but, like for PSI-D, the highest amounts were found in older leaf sections.

Discussion

The light- and developmental-dependent expression of proteins involved in chloroplast metabolism has been studied extensively with two experimental systems, the etioplast-chloroplast conversion and the developmental gradient within leaves of monocotyledonous cereals (Leech, 1982; Martineau and Taylor, 1985; Takabe *et al.*, 1986). A direct comparison of these experiments is diffcult, because different plant species were used and different proteins were analysed. However it has been shown in other studies, that chlorophyll has to be present for the accumulation of the chlorophyll-binding subunits of both photosystems (Kreuz *et al.*, 1986; Sutton *et al.*, 1987). In agreement with these findings are experiments which demonstrate that the photosynthetic electron transport chain develops gradually. In general, photosystem I activity can be observed before a functional photosystem II is formed, and the whole electron transport from water to NADP reaches it's full activity only several hours after the illumination of etioplasts (Wellburn, 1982; Baker, 1984; Ohashi *et al.*, 1989). On the other hand, it has long been known that the ATPase is present in etioplasts (Lockshin *et al.*, 1971; Wellburn, 1977), but no functional importance has been ascribed to this observation.

We have extended these studies to another protein complex of the thylakoid membrane, the NAD(P)H-plastoquinone oxidoreductase. We found that the subunits NDH-H and NDH-K accumulate in etioplasts to high amounts. Moreover these proteins become synthesized at the earliest stages of natural plastid development in the light. Etioplasts and developing plastids are "heterotrophic" organelles and rely on the import of energy and carbon skeletons for growth, which is mediated by specific carriers in the plastid envelope (Wellburn, 1982; Flügge and Heldt, 1991). Etioplasts and developing chloroplasts build up voluminous internal membrane systems, the prolammellar body with protruding prothylakoids and the thylakoids, respectively. This development of internal membranes requires the synthesis of nuclear and plastid encoded proteins, the import of cytoplasmatically synthesized proteins into the organelle and the insertion of proteins into the prothylakoid or growing thylakoid membranes. All of these processes are dependent on an sufficient energy supply in the form of ATP (De Boer and Weisbeek, 1991; Cohen *et al.*, 1995). However, the integration or translocation of certain proteins into the growing thylakoid membrane requires in addition a proton gradient across the membrane. This has been shown for example for the light-harvesting chlorophyll *a/b*-binding proteins (LHCP), the Rieske iron-sulfur protein and the

subunits of the so-called oxygen-evolving subcomplex of photosystem II (Cline *et al.*, 1992; Cohen *et al.*, 1995). On the other hand, etioplasts and developing chloroplasts posess no functional photosynthetic electron transport chain and are thus not able to build up a proton gradient by light-driven electron transport. Our experiments show that the NAD(P)H-plastoquinone-oxidoreductase and the ATPase are the only major protein complexes of the prothylakoid membrane. We therefore suggest that the NAD(P)H-plastoquinone-oxidoreductase by oxidizing reduction equivalents imported into the etioplast generates a proton gradient and enables the integration and the assembly of internal thylakoid membrane proteins. A functional ATPase would then be required for the counterregulation of this gradient.

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