In vitro import of pre-ferredoxin-NADP$^+$-oxidoreductase from *Cyanophora paradoxa* into cyanelles and into pea chloroplasts**

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Abstract Using a novel cyanelle isolation procedure we showed that pre-ferredoxin-NADP$^+$-oxidoreductase (pre-FNR) from *C. paradoxa* is translocated in vitro across the peptidoglycan-containing cyanelle envelope. Efficient import was also observed in a heterologous system with pea chloroplasts as the recipient organelles. These results support the conclusion derived from comparative analysis of plastid genome organization, that all plastids originate from a common semi-autonomous endosymbiotic ancestor.

Key words: Pre-ferredoxin-NADP$^+$-oxidoreductase; Import, in vitro; Cyanelles; *Cyanophora paradoxa*; Pea chloroplast; Plastid evolution

1. Introduction

Cyanophora paradoxa is a photoautotrophic fresh-water protist harboring plastids (cyanelles) that have retained a prokaryotic cell wall between their two envelope membranes [1–3]. The cyanelles as well as the rhodoplasts from the red alga *Porphyra purpurea* [4] encode far more proteins than the chloroplasts of higher plants and are considered to be ancient plastid types. Comparative analysis of plastid genome organization led to the conclusion of a singular primary endosymbiotic event, i.e. that all plastids regardless of their pigment composition and morphology are derived from a common precursor organelle [2,4–6]. The plastids of euglenoids, chromophytes, and cryptophytes arose from superimposed secondary endosymbiotic events involving eukaryotic algae engulfed by heterotrophic secondary host cells [6]. Support for the concept of a common origin of plastids could also come from a comparison of their protein import apparatus. In vivo experiments indicated that the majority of cyanelle polypeptides has to be imported from the cytoplasm [7]. The FNR precursor of *C. paradoxa* has to cross two membranes and the peptidoglycan layer. Its transit sequence resembles the stroma targeting peptides of chloroplast-located, nucleus-encoded proteins from higher plants [8]. In vitro import of precursor polypeptides into isolated cyanelles has not been achieved to date. In this paper we describe the homologous import of pre-FNR in vitro as well as the heterologous import into isolated pea chloroplasts. In addition the inverse heterologous import of pre-FNR from the higher plant *Mesembryanthemum crystallinum* into isolated cyanelles is demonstrated.

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**The authors wish to dedicate this paper to Professor Helmut Kindl on the occasion of his sixtieth birthday.

2. Experimental

2.1. Isolation of import-competent cyanelles

C. paradoxa 555 UTEX was grown as described [9]. Cells were harvested, washed once and then suspended in 10×BSM (isoosmotic buffered sugar mixture, pH 8.0) containing the main sugar metabolites in *C. paradoxa* and the osmotica protective for cyanelle integrity [10]. 50×BSM stock: 0.33 M sucrose, 0.11 M maltose, 0.4 M glucose, 0.16 M mannitol, 0.77 M betaine, and 1.15 M glycerol. After 10 min on ice the suspension was centrifuged for 5 min at 450×*g* at 4°C. For cyanelle isolation the cells were exposed to an osmotic shock in 1.3×BSM and were passed three times through a Potter homogenizer. Separation of cyanelles and cell fragments was performed by differential centrifugation in 50 ml Falcon tubes for 10 min at 100×*g*. The milky supernatant (40 ml) and the flocculent layer of cell fragments and intact cells (about 5 ml) above the cyanelle pellet were discarded. The material at the bottom of the tube (1 ml) was resuspended in 1.3×BSM. Two more washing steps were necessary to obtain a cyanelle fraction essentially free of cytosolic contamination. During this process no leakage of cyanelles (as indicated by the blue color of the supernatant) was observed. The isolated cyanelles were suspended in import buffer (IB; 50 mM L-methionine, 1 mM ATP, 1 mM ε-aminocaproic acid, 0.1 mM Pefabloc (Boehringer) in 1×BSM, 10 mM HEPES pH 7.5). 300 μl of this suspension and 4 μl of the translation mixture were used for the import experiments. Incubations were performed for one hour under slight shaking in order to prevent the cyanelles from sedimentation. Cyanelles were then isolated again by centrifugation for 2 min at 1,000 rpm in an Eppendorf centrifuge and washed twice with IB. Thermolysin treatment: thermolysin (100 μg/ml) was added to IB containing 10 mM $CaCl_2$ and the resuspended cyanelles were incubated for 30 min at 4°C. Aliquots of the different experiments were analysed on 12% discontinuous SDS-PAGE.

2.2. Import into isolated pea chloroplasts

Chloroplasts were isolated from 14-day-old pea leaves as described elsewhere [11].

2.3. Preparation of labelled precursor

In vitro transcription was performed using T3-polymerase (Boehringer) on a linearized plasmid template. To increase the translation efficiency, we used a modified Bluescript vector, pBAT, containing an insert of 55 bp from the rabbit β-globin leader region [12]. An *Eco*RI fragment containing the pre-FNR cDNA from *C. paradoxa* [8] and an *Eco*RV fragment containing the counterpart from *Mesembryanthemum crystallinum* [13], respectively, were cloned into the polylinker. The mRNA was purified by phenol extraction and precipitation in high salt/ethanol. For translation using reticulocyte lysate (Promega) the template was diluted to a final concentration of 0.8 ng/ml. 40 mCi of [^{35}S]methionine (Amersham) were used for radiolabelling.

3. Results

Isolation methods applicable to obtain intact, transport-

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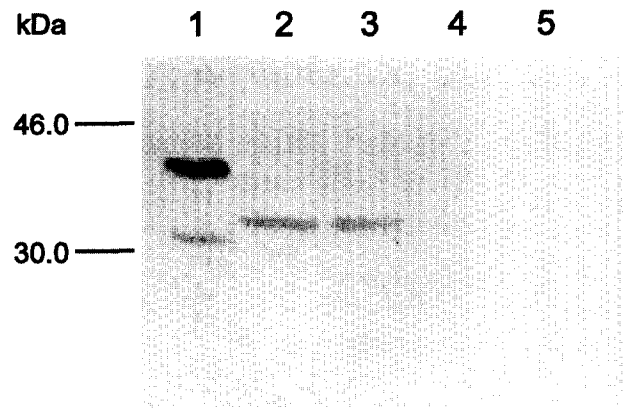

Fig. 1. Import of pre-FNR from *C. paradoxa* into cyanelles contained within a whole cell lysate. Lane 1 = translation mix; lane 2 = incubation at 25°C in the light followed by thermolysin treatment; lane 3 = w/o thermolysin treatment; lane 4 = incubation at 4°C in the dark followed by thermolysin treatment; lane 5 = w/o thermolysin treatment.

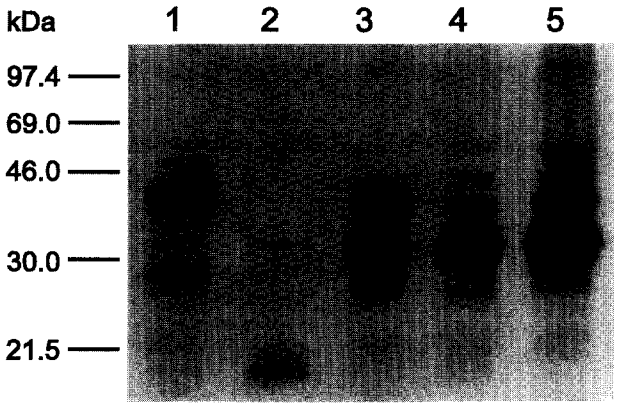

Fig. 3. Heterologous import of pre-FNR from *C. paradoxa* into isolated pea plastids. Lane 1 = translation mix; lane 2 = incubation at 4°C in the dark followed by thermolysin treatment; lane 3 = w/o thermolysin treatment; lane 4 = incubation at 25°C in the light followed by thermolysin treatment; lane 5 = w/o thermolysin treatment.

competent chloroplasts of higher plants cannot be used to collect physiologically intact cyanelles [2]. Failure to import higher plant precursors into isolated cyanelles (S. Smeekens and M. Brandtner, unpublished) was ascribed to lack of competence of cyanelles isolated by conventional methods. In a homogenate of broken *Cyanophora* cells containing cyanelles and cytoplasm low efficiency import of the homologous FNR precursor could be observed (Fig. 1). The homogenate proved to be rich in proteases. Only the internalized and processed mature FNR was protected, regardless if additional thermolysin treatment took place. Data from metabolite exchange studies [10,14] made it possible to simulate a 'cytosolic' environment for cyanelles during the isolation steps and to establish genuine in vitro conditions. Using BSM, cyanelles could be held stable for several hours. However, the cyanelles prepared by this method still show only low import competence (Fig. 2) compared to pea chloroplasts (Fig. 3) that recognized and imported the heterologous precursor with high efficiency. In addition to specific processing of the 67 amino acid transit peptide [8], converting the 41 kDa precursor into the 34 kDa mature protein, unspecific degradation was also observed (Fig. 2, lane 5; Fig. 3, lane 3). The degradation products were not protected from the action of thermolysin. At 4°C in the dark only binding occurs but not translocation as it is also observed with chloroplasts [16,17]. The FNR precursor from the higher plant *M. crystallinum* was also imported into isolated cyanelles, although with low efficiency (Fig. 4). Thus, the *Cyanophora* precursor is recognized by the import receptors of higher plant chloroplasts, is translocated and processed, and vice versa. The peptidoglycan wall is no barrier for the continuous passage of more than 800 polypeptides. In order to exclude dependence of the import on a diurnal cycle or on the stage of the cell cycle, *Cyanophora* cells were isolated at different times during the light/dark period (ranging from 12/12 to 16/8 h). No dependence was observed.

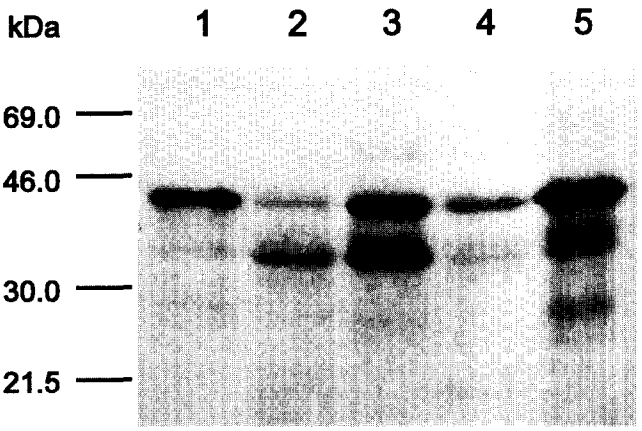

Fig. 2. In vitro import of pre-FNR from *C. paradoxa* into cyanelles isolated using the BSM. Lane 1 = translation mix; lane 2 = incubation at 25°C followed by thermolysin treatment; lane 3 = w/o thermolysin treatment; lane 4 = incubation at 4°C followed by thermolysin treatment; lane 5 = w/o thermolysin treatment.

4. Discussion

Transit peptide sequences of nuclear genes for cyanelle proteins function in heterologous import assays into higher plant chloroplasts. To a lesser extent this is also the case for stroma targeting peptides from higher plant precursors with respect to import into isolated cyanelles. These parallels may allow conclusions about an ancestral import system which must have evolved prior to the event that led to cyanelles. The ancestral semi-autonomous endosymbiont, most likely, was still surrounded by a peptidoglycan layer. This cyanobacterial-type wall became reduced in thickness as it is the case in the cyanelle wall and likely became modified to facilitate translocation of proteins. Cyanelle peptidoglycan is substituted with *N*-acetylputrescine at C-1 of the D-glutamyl residue, while peptidoglycan from the cyanobacterium *Synechocystis* 6714 does not contain this modification [2]. Recently, the transit peptide of γ-phycoerythrin from the red alga *Aglaothamnion neglectum* was shown to direct the import of a passenger protein into pea chloroplasts [18]. Only rhodoplasts, cyanelles and chloroplasts of green algae and higher plants are surrounded by two membranes which is the signature of the ancient primary endosymbiotic event, irrespective of the evolutionary distance between these groups. These three lines evolved directly from the semi-autonomous endosymbiotic ancestor. The additional

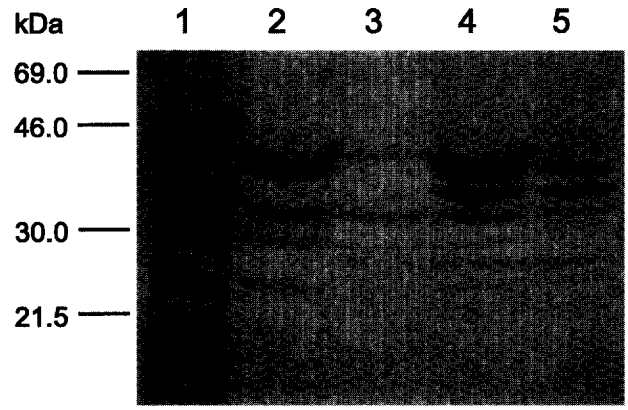

Fig. 4. Heterologous import of pre-FNR from *M. crystallinum* into isolated cyanelles. Lane 1 = translation mix; lane 2 = incubation at 4°C in the dark w/o thermolysin treatment; lane 3 = followed by thermolysin treatment; lane 4 = incubation at 25°C in the light w/o thermolysin treatment; lane 5 = followed by thermolysin treatment.

envelope membranes found in the plastids of euglenoid algae, chromophytes and cryptophytes show characteristics of endoplasmic reticulum-derived membranes. This seems to have necessitated different targeting signals and more complex import mechanisms. Although there is only little information, the available data indicate that the import signals in these groups are bi-partite. An amino terminal signal sequence for co-translational translocation through the periplastid reticulum membranes is found which precedes a chloroplast-type transit peptide sequence of variable length [19–21]. The opposite organization is observed in the bi-partite targeting signals of nucleus-encoded proteins located in the thylakoid lumen that are transported as predicted by the 'conservative sorting' hypothesis [22].

Inner and outer bacterial membranes are connected by a lipoprotein embedded in the outer membrane which is covalently linked to the peptidoglycan layer. This lipoprotein is missing in cyanelles, which is most probably the reason for the instability of the outer envelope membrane during isolation [23]. When isolated by conventional methods, partial loss of the outer membrane seems to be the cause for the poor performance of isolated cyanelles in CO_2 fixation and protein synthesis [2], although these cyanelles appear intact upon phase contrast microscopy. Conventionally isolated cyanelles are not competent at all to import heterologous pre-proteins (S. Smeekens, personal communication), nor is the transport of homologous pre-proteins supported (J. Jakowitsch, unpublished). Further optimization will be necessary to improve the import efficiency of cyanelles prepared using BSM and to make it comparable to that of higher plant plastids. It is our opinion that the poor performance of isolated cyanelles in the import of higher plant pre-FNR even in the novel medium does not reflect the heterologous conditions *per se* or pronounced differences between the import apparatus of chloroplasts and cyanelles. It will be interesting to compare the cyanelle envelope proteins with the import receptors which have recently been identified in higher plant chloroplasts [15,24,25].

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