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Localization and processing of pD1 in *Synechocystis* PCC6803 membrane fractions

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Introduction

The cyanobacterium *Synechocystis* PCC6803 (*S*. 6803) is a photoautotrophic organism, also capable of heterotrophic growth. Photosynthetic reactions take place in thylakoid membranes. Three *psbA* genes encode PSII reaction center core protein D1 in *S.* 6803, of which *psbA2* and *psbA3* genes produce identical D1 proteins (Ravnikar *et al*., 1989; Metz *et al*., 1990). *psbA1* gene is not transcribed under typical laboratory conditions (Mohamed and Jansson, 1989). As with higher plants, also the cyanobacterial D1 protein is translated as a precursor protein (pD1) with a conserved carboxy terminal extension of 16 amino acids, the length being typical for cyanobacteria (Igleva *et al*., 2000). The function of the extension is unknown and mutants lacking it are able of autotrophic growth. pD1 is co-translationally inserted into PSII complexes in thylakoid membranes (Zhang, *et al*., 1999) and further posttranslationally processed to mature form by the lumenal protease CtpA (Shestakov *et al*., 1994).

This study is based on immunological analysis of isolated pure *S*. 6803 membrane fractions obtained from 2D-isolation of plasma and thylakoid membranes (Norling *et al*., 1998). Different photosynthetic polypeptides were identified from these membrane fractions. Purity of fractions was verified with specific marker proteins. D1 protein is the only PSII core protein translated with a precursor sequence. To reveal the role of different membrane types in synthesis of D1 protein during it's light activated turnover, we have simply examined the location of precursor form of D1 protein in different membrane fractions.

Materials and methods

S. 6803 cells were grown photoautotrophically in BG-11 medium at 30 °C under 50 µmol photons $m^{-2}s^{-1}$ of white light and constant air bubbling. The cells were harvested when chlorophyll concentration reached 10 µg/ml. Inhibition of translation initiation was obtained by adding lincomycin (Sigma) to the growth media to a final concentration of 400 µg/ml, when indicated, one hour before harvesting. A modified version of the method of 2D-isolation of pure plasma and thylakoid membranes (Norling *et al*., 1998) was employed. Here we first separated membranes according to density (sucrose density centrifugation) and thereafter according to surface properties (two phase partitioning). The scheme of low temperature photoinhibition (Kanervo *et al*., 1997) was employed in radioactive labeling of *S.* 6803 proteins. Crude thylakoid isolation was performed as described previously (Gombos *et al*., 1994). Polypeptide separation, western blotting and autoradiography were performed according to standard procedures. Blots were probed with antibodies raised against carboxy terminal extension of D1, D-E-loop of D1, D-E-loop of D2, CP43, NrtA and cytb559. In western blots of purified membrane fractions, 20 µg protein was applied to each well or 3 µg

chlorophyll when crude thylakoid fractions were analyzed.

Results

The purity of membrane subfractions of *S.* 6803 was verified by marker proteins NrtA (periplasmic binding protein of the nitrate transporter), residing only in the plasma membrane and CP43 (chlorophyll-a binding protein of PSII) a specific marker for the thylakoid membranes (Norling *et al*., 1998). Fig 1. illustrates the degree of cross contamination between plasma membrane and thylakoid membrane of the final subfractions obtained from two phase partitioning. Fig 1. further shows that PSII core proteins D1, D2 and $cytb_{559}$ are mainly found in thylakoids but importantly, small amounts exist in plasma membrane as well.

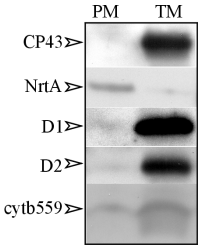

Fig 1. Immunoanalysis of membrane preparations obtained from *S.* 6803 after 2D-isolation of plasma (PM) and thylakoid membrane (TM). Existence of CP43 only in thylakoid and NrtA only in plasma membrane indicate purity of membrane fractions.

Probing the membranes with an antibody raised against the carboxy terminal extension of D1 showed pD1 to reside exclusively in the thylakoid membrane (Fig. 2A).

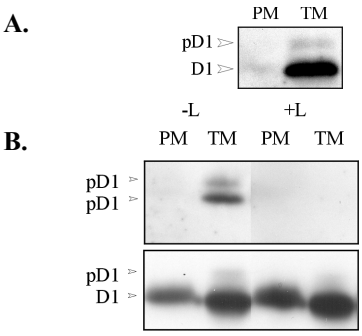

Fig 2. Localization of pD1 in membrane fractions. **A**. Immunoblot demonstrating pD1 and mature D1 in *S.* 6803 thylakoid membrane fraction. **B**. Immunoblot demonstrating the effect of lincomycin (+/- L) on accumulation of pD1 under growth conditions. Interestingly, here pD1 can be clearly observed as two distinct bands.

The membrane was thereafter probed against mature form of D1 with a D-E-loop specific antibody. D-E-loop antibody is not sensitive enough to recognize pD1. Adding lincomycin (1 h treatment) to the cell culture under normal growth conditions inhibited translation initiation. Incubation of cells in presence of lincomycin-induced disappearance of pD1 (Fig. 2B) whereas no clear decrease was seen in mature D1 protein during incubation at this low light. In order to accumulate pD1 protein we performed low temperature photoinhibition (Kanervo *et al*., 1997) with *S.* 6803 followed by labelling of cells with ^{35}S-L-Met. Under these conditions, ^{35}S-L-

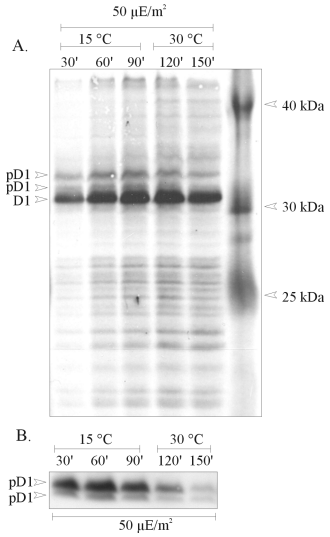

Fig 3. Autoradiogram (**A.**) and immunodetection (**B.**) of D1/pD1 in crude thylakoid preparations. After low temperature photoinhibition treatment (1500 µE/m^2, 15 °C, 1 h) cells were labeled with ^{35}S-L-Met at low light and low temperature (50 µE/m^2, 15 °C) for 30', 60' and 90'. Cells were then transferred to normal growth conditions for further 30' and 60' (referring to 120' and 150' respectively) inducing maturation of D1 by C-terminal processing (**A.**).
B. Same samples immunoblotted with pD1 specific antisera.

Met was mostly incorporated to D1 (Fig. 3A) and precursor form of D1 became also labelled. Transfer of cells to normal growth conditions induced a disappearance of pD1. The same samples as used for autoradiograpy in Fig. 3A were further used in western blotting with anti pD1 antibody (Fig. 3B). Interesting enough, processing of precursor peptide seemed to occur in two steps, an indication of two different cleavage sites in the extension (Fig. 2B and Fig. 3A and 3B).

Discussion

Traces of PSII core proteins were found in the plasma membrane of *Synechocystis* PCC6803 the majority being in thylakoids. The role of PSII proteins in plasma membrane, however still remains unknown. Photosynthesis components (chlorophyll, chlorophyll precursors and photosynthesis machinery proteins) have been found not only in thylakoid membranes but also in plasma membrane of cyanobacteria. (Norling *et al*., 1998; Peschek *et al*., 1989; Smith *et al*., 1992; Smith and Howe, 1993; and Sherman *et al*., 1994). Considering our results and the earlier findings, it could be postulated that plasma membrane may act as a site of PSII core protein synthesis in cyanobacteria.

One approach to address the question of plasma membrane as a possible initial synthesis site for photosynthetic core proteins, is to localize pD1. D1 is synthesized as a precursor form with a carboxy terminal extension of 16 amino acids (Igleva *et al*., 2000). We have examined the role of plasma membrane in synthesis of PSII core proteins by localizing pD1 in different membrane subfractions obtained from 2D-isolation. Further investigations with pD1 antibody showed a band in thylakoid membrane fraction with a molecular mass slightly higher than that of mature D1 protein (Fig. 2A). However, the amount of mature D1 is so low in plasma membrane that possible preforms of D1, if present in similar ratio to mature D1 as in thylakoid membranes, cannot be detected by our immunoblotting approaches. Although the pD1 amount is low under growth conditions in thylakoid membranes, it can be immunologically identified. To induce accumulation of pD1 in *S*. 6803, we treated cells under low temperature photoinhibition conditions. Subsequently ^{35}S-L-Met was fed to the cells. Autoradiogram (Fig. 3A) of protein blots enabled visualization of both pD1 and D1. Same samples, as visualized in autoradiogram, were used to perform western blotting with anti-pD1 antibody. pD1 started accumulating when cells, after photoinhibition treatment, were subjected to radiolabelling at 15 °C and 50 $\mu E/m^2$. C-terminal processing of pD1 by lumenal CtpA was inefficient at 15 °C. Transfer of cells to 30 °C induced efficient processing of pD1. Fig. 3B demonstrates our observation of two possible cleavage sites in pD1 protein or alternatively migration of pD1 as two distinct bands can be explained by two different conformations of pD1.

Taken together, the relation between PSII core proteins in the thylakoid and plasma membrane needs further investigation considering aspects of PSII biogenesis in *S*. 6803. However, our results seem to exclude plasma membrane as D1 synthesis site when photodamaged D1 protein is replaced in PSII centers with *de novo* synthesized D1 copy during the repair cycle. Immunological identification of the precursor form of D1 protein localized pD1 in thylakoid membrane fraction only.

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