Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1767 (2007) 829–837
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbabio

Cleavage after residue Ala352 in the C-terminal extension is an early step in the maturation of the D1 subunit of Photosystem II in *Synechocystis* PCC 6803

Josef Komenda [a,b,*], Stanislava Kuviková [a,b], Bernhard Granvogl [c], Lutz A. Eichacker [c], Bruce A. Diner [d], Peter J. Nixon [e]

[a] *Institute of Microbiology, Academy of Sciences, Opatovický mlýn, 37981, Třeboň, Czech Republic*
[b] *Institute of Physical Biology, University of South Bohemia, Zámek 136, 37333, Nové Hrady, Czech Republic*
[c] *Botanical Institute, Ludwig-Maximilian University Munich, Menzinger Str. 67, 80368 Munich, Germany*
[d] *Experimental Station, E. I. du Pont de Nemours & Co.,Wilmington, DE 19880-0173, USA*
[e] *Wolfson Biochemistry Building, Division of Biology, Faculty of Natural Sciences, Imperial College London, SW7 2AZ, UK*

Received 25 September 2006; received in revised form 7 January 2007; accepted 8 January 2007
Available online 17 January 2007

Abstract

We have investigated the pathway by which the 16 amino-acid C-terminal extension of the D1 subunit of photosystem two is removed in the cyanobacterium *Synechocystis* sp. PCC 6803 to leave Ala344 as the C-terminal residue. Previous work has suggested a two-step process involving formation of a processing intermediate of D1, termed iD1, of uncertain origin. Here we show by mass spectrometry that a synthetic peptide mimicking the C- terminus of the D1 precursor is cleaved by cellular extracts or purified CtpA processing protease after residue Ala352, making this a likely site for formation of iD1. Characteristics of D1 site-directed mutants with either the Leu353 residue replaced by Pro or with a truncation after Ala352 are in agreement with this assignment. Interestingly, analysis of various CtpA and CtpB null mutants further indicate that the CtpA protease plays a crucial role in forming iD1 but that, surprisingly, low levels of C-terminal processing occur in vivo in the absence of CtpA and CtpB, possibly catalysed by other related proteases. A possible role for two-step maturation of D1 in the assembly of PSII is discussed.

Keywords: CtpA protease; D1 maturation; Photosystem II; *psbA* gene; *Synechocystis* PCC 6803

1. Introduction

The D1 protein is a key integral protein subunit of Photosystem II (PSII), the thylakoid pigment–protein complex responsible for water oxidation and release of oxygen in plants, algae and cyanobacteria [1]. In the cyanobacterium *Synechocystis* 6803, D1 is encoded by two functional genes, *psbA2* and *psbA3* [2], and is synthesized as a precursor (pD1) with a carboxyl-terminal extension consisting of 16 amino-acid residues [3]. The precursor is subsequently cut on the carboxyl side of residue Ala344 by a specific processing endoprotease CtpA [4] resulting in the removal of the extension [5]. This removal is a prerequisite for the assembly of manganese cluster and is therefore essential for a fully functional PSII complex. Roose and Pakrasi [6] recently suggested that cleavage of the extension is also needed for binding of the PSII extrinsic proteins into PSII. Following the radioactive labeling of *Synechocystis* cells at low temperature, Inagaki et al. [7] detected an intermediate D1 band (iD1) upon SDS-PAGE that could represent a partially truncated form of pD1. They therefore suggested that unlike the D1 protein in higher plants,

Abbreviations: Chl, chlorophyll; DM, dodecylmaltoside; HRA, Hill reaction activity; LIN, lincomycin; PS, Photosystem; MS, mass spectrometry; PSII, photosystem II

* Corresponding author. Institute of Microbiology, Division of Autotrophic Microorganisms, Academy of Sciences, Opatovický mlýn, 37981 Třeboň, Czech Republic. Fax: +42 384 721 246.
E-mail address: komenda@alga.cz (J. Komenda).

0005-2728/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2007.01.005

the cyanobacterial version is cleaved in two consecutive steps with the primary cut occurring close to the middle of the extension. Unambiguous evidence to support this double-cleavage mechanism, including the precise localization of the primary cleavage site, is however still lacking.

In this manuscript, we have focused on the identification of the site of pD1 cleavage leading to the formation of intermediate iD1. We have obtained evidence that iD1 originates from cleavage of the D1 precursor after residue Ala352. This cut is not essential for the final cleavage step after residue Ala344, although PSII in the mutant unable to perform the maturation step after Ala352 exhibited a slightly increased sensitivity to photoinhibition. Surprisingly, analyses of strains lacking CtpA and CtpB processing proteases indicated the possible participation of additional proteases in D1 maturation, although at physiologically insignificant rates.

2. Materials and methods

2.1. Strains and culture conditions

The following mutants of the glucose-tolerant strain *Synechocystis* sp. PCC 6803 referred to as wild-type (WT) [8] were used in the study: (i) the CtpA-less strain ΔCtpA with the *ctpA* gene inactivated by an spectinomycin (*spec*^{R}) resistance cassette; (ii) the CtpA-less and CtpB-less strain ΔCtpA/ ΔCtpB with the *ctpA* and *ctpB* genes inactivated by *spec*^{R} and kanamycin (*kan*^{R}) resistance cassettes, respectively; (iii) the D1-less strain, ΔD1, with the *psbA1*, *psbA2* and *psbA3* genes inactivated by chloramphenicol (*cm*^{R}), *kan*^{R} and *spec*^{R} resistance cassettes, respectively [9]; (iv) the site-directed mutant, D1-Ser345Pro, having the *psbA1* and *psbA2* genes inactivated by *cm*^{R} and *kan*^{R} cassettes, respectively, and the codon for residue Ser345 in the *psbA3* gene replaced by a codon for Pro [5] and (v) the corresponding D1-Ser345Pro/ ΔCtpA mutant in which the *ctpA* gene was disrupted through insertion of *spec*^{R} cassette. The strains were grown in BG-11 medium containing 5 mM glucose, the newly constructed autotrophic strains were grown without glucose. Solid media contained in addition 10 mM TES/NaOH, pH 8.2, 1.5% agar and 0.3% sodium thiosulphate [10]. 50–100 ml liquid cultures were shaken in 250 ml conical flasks at 29 °C with a surface irradiance of 30 μmol photons m^{-2} s^{-1} of white light. Cultures were analyzed when in the late exponential phase (chlorophyll concentration approx. 5 μg ml^{-1}) or, in the case of autotrophic strains, they were transferred in this phase into double-wall, thermoregulated cultivation cylinders (internal diameter 35 mm). Here, the culture was maintained at a chlorophyll concentration of 6–8 μg ml^{-1} by regular diluting with approx. 10 ml of BG-11 medium every 150 min and this culture was used for characterization of growth and photosynthetic properties. The culture was bubbled with air containing 2% (v/v) CO_2 and illuminated with white light at 40 μmol photons m^{-2} s^{-1} and 29 °C.

2.2. Construction of D1 site-directed mutants by megaprimer mutagenesis

Site-directed mutagenesis of the *psbA2* gene was based on PCR megaprimer method using two rounds of PCR that employ two flanking primers with different melting temperatures and one internal mutagenic primer [11]. The resulting 1.8-kbp PCR product containing the mutated *psbA2* gene with upstream and downstream regions was directly used to transform the *psbA* triple-deletion strain [9]. Autotrophic transformants expressing the D1 protein with modified C-terminal extension were selected on BG11 plates without glucose. The control strain, A2, expressing the wild-type form of D1 was obtained by a transformation of the *psbA* triple deletion strain using the same 1.8-kbp PCR product without mutation. Sequencing of the entire *psbA2* region amplified by PCR confirmed that in all strains the particular gene has been correctly inserted under the control of the *psbA2* promotor.

2.3. Steady-state rate of oxygen evolution

Light-saturated steady-state rate of oxygen evolution in cell suspensions was measured at 29 °C using a Clark-type electrode (YSI, USA) in the presence of artificial electron acceptors p-benzoquinone (0.5 mM final concentration) and potassium ferricyanide (1 mM final concentration) as described in [9].

2.4. Thylakoid preparation and protein analyses

Thylakoid membranes were prepared by breakage of the cells with glass beads (150–200 μm in diameter) at 4 °C followed by differential centrifugation as described in [12]. The protein composition of thylakoids was assessed by electrophoresis in a denaturing 12–20% linear gradient polyacrylamide gel containing 7 M urea [13]. Proteins were electroblotted from gel onto PVDF membrane (Amersham Biosciences) and detected using D1-specific antibodies raised against amino-acid residues: (i) 2–17 of *Synechocystis* D1 (anti-D1(N)); (ii) 59–76 of the *Synechocystis* D1 (anti-D1(M)); and (iii) 345–360 of the *Synechocystis* D1 (anti-pD1) [5].

2.5. Incubation of the synthetic peptide either with cellular extract from the Synechocystis strains or with purified CtpA protease from Scenedesmus

Cells of wild-type and mutants ΔCtpA and ΔCtpA/ΔCtpB (approximately 0.5 mg of chlorophyll) were resuspended in 2 ml screw-capped eppendorf tube in 0.5 ml of 25 mM HEPES/NaOH, pH 7.5 and 0.5 ml of glass beads (150–200 μm in diameter) were added. The cells were broken in Minibeadbeater (Biospec. Products, USA) five times 20 sec with 2 min interruption for cooling in ice. Suspension was quickly spun down in the Minispin centrifuge (Eppendorf, Germany) at 8 000 rpm for 1 min and the supernatant was removed. 225 μl of the supernatant was mixed with 25 μl of 10% dodecyl maltoside and mixture was spun down at 13 000 rpm for 5 min. Supernatant was mixed with 25 μl of 10 μM synthetic peptide HHHHHHNFPLDLAPGE-QAPVALTAPAVNG (Clonestar Peptide Services, Czech Republic) and incubated at room temperature for 15 h with a regular moderate mixing. Then the mixture was added to 0.25 ml of Ni-Fractogel EMD Chelat (Merck, Germany) equilibrated with 25 mM MES/NaOH, pH 6.5 containing 100 mM NaCl and 0.04% dodecylmaltoside in 0.5 ml eppendorf tube. After 5 min incubation the bottom of the eppendorf tube was pierced, tube was placed into 1.5 ml tube and spun down at 5 000 rpm for 10 s. Then the dry Fractogel was washed five times with equilibration buffer containing 1 mM imidazole and finally the bound peptides were released by 200 μl of 200 mM imidazole. If purified CtpA protease from *Scenedesmus obliquus* [14] was used instead of the cellular extract, the incubation was performed in the presence of 25 mM HEPES/KOH, pH 7.5 containing 1 mM peptide and 20 μg ml^{-1} of enzyme for 15 h at room temperature.

2.6. Mass spectrometry of the peptides

Prior to analysis, salts and detergents in the sample were removed by adsorbing the peptides to a ZipTip^{TM} C_{18}-reversed phase microcolumn (Millipore, USA) according to the manufacturer's instructions with exception that in all solutions acetonitrile and trifluoroacetic acid was replaced by methanol and formic acid to obtain ESI-spectra of better quality. All mass spectrometric analyses were carried out on a Q-TOF-I hybrid mass spectrometer (Micromass, UK), equipped with an orthogonal electrospray ionization source operated in the positive electrospray ionization mode (ESI+). Mass accuracy was ±0.1 Da.

2.7. Chlorophyll content

For measurement of chlorophyll concentrations, cells were sedimented by centrifugation and extracted with 100% methanol. The concentration of chlorophyll was calculated from the absorbance values of the extract at 666 and 720 nm [15].

3. Results

3.1. Mass spectrometric analysis of an in-vitro cleaved synthetic peptide indicates CtpA-specific cleavage of the extension after Ala352

The detection by SDS-PAGE of an intermediate form of D1, designated iD1, in the cyanobacterium *Synechocystis* has led to the hypothesis that the C-terminal extension of pD1is removed in two stages [7]. The intermediate, iD1, has subsequently been detected in other studies [9,13,16] but its origin has remained uncertain. Based on the comparison of electrophoretic mobilities of pD1, iD1 and D1, Inagaki at al. [7] proposed that the initial step might occur in the middle of the extension. As suggested by the final cleavage of pD1 after Ala344 [5], we speculated that the processing protease CtpA might specifically cleave the extension after Ala residues and therefore we proposed that the cleavage site lay after residue Ala352, i.e. precisely in the middle of the extension. To test this idea we first performed an in-vitro cleavage assay using a synthetic oligopeptide containing: (i) residues His337-Gly360 of the *Synechocystis* D1 precursor; (ii) a penta His-tag attached to the N-terminus of the peptide; and (iii) the Pro residue at the position 345 instead of the original Ser (Fig. 1A). The penta His-tag was attached to the peptide in order to purify the N-terminal peptide fragments after cleavage. The replacement of Ser345 for Pro was introduced in order to inhibit the final cleavage of the extension [5]. In this way, sufficient amounts of N-terminal fragment(s) originating from the primary cleavage and containing the penta His-tag could be isolated and characterized. We incubated the peptide with a dodecylmaltoside-treated extract of *Synechocystis* wild-type cells for 15 h at room temperature. From this extract, the peptides containing the penta His-tag were bound to Chelating Fractogel containing immobilized Ni^{2+} ions. Peptides bound to the Fractogel were released by 200 mM imidazole and the eluate was analyzed by an electrospray ionization mass spectrometer using Q-TOF. The spectrum of the original peptide was characterized by two large signals

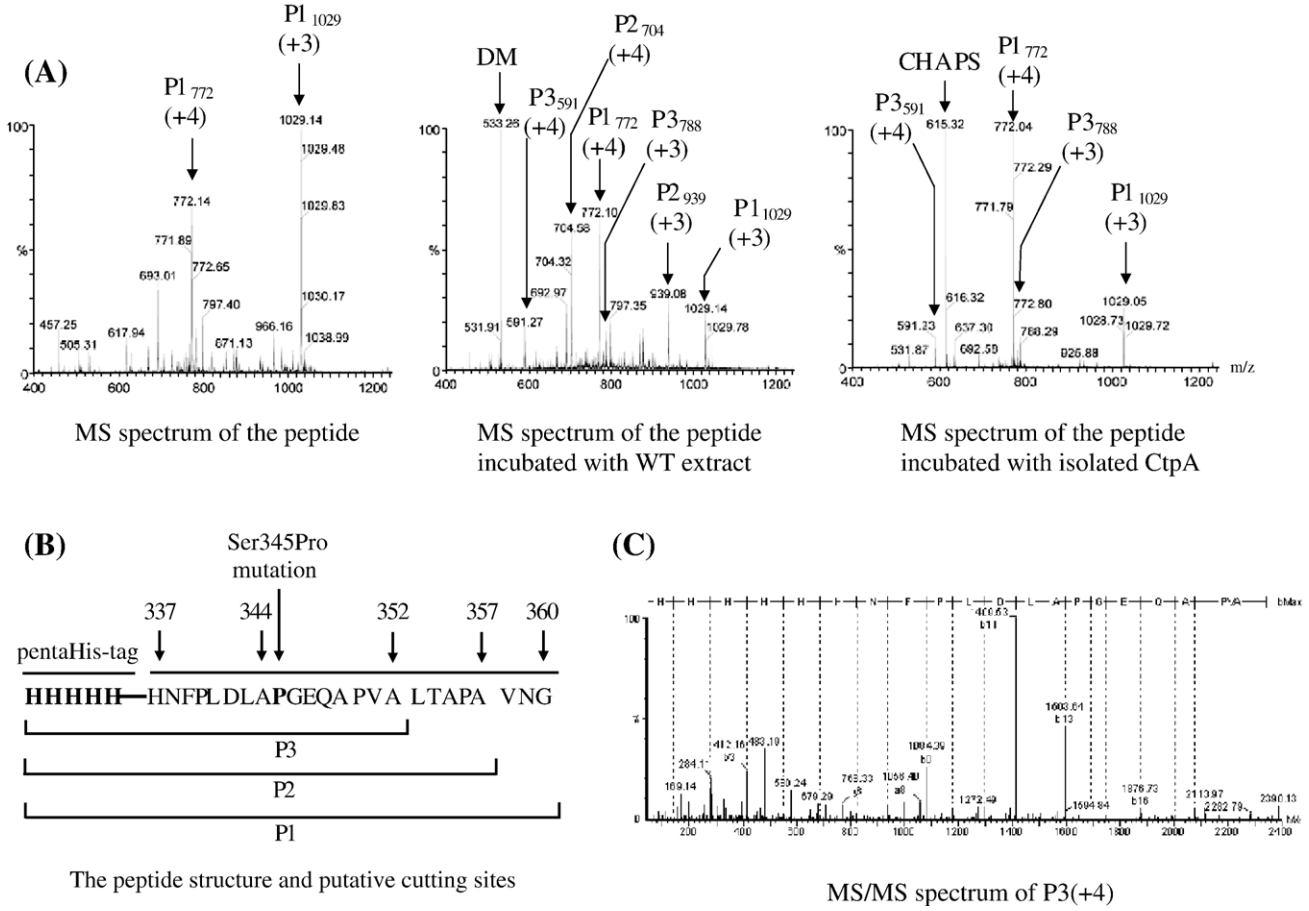

Fig. 1. Mass spectrometric analysis of the synthesized peptide derived from the C-terminal sequence of the D1 precursor before and after incubation with the extract of the *Synechocystis* wild-type cells or with the purified CtpA protease (A), sequence of the peptide and its putative cleavage sites (B) and MS/MS analysis of the P3(+4) signal (C). (A) MS-signals of the synthesized peptide derived from pD1 sequence His337–Gly360 before (left panel) and after incubation with WT cellular extract (middle panel) or purified CtpA protease (right panel). In Nano-ESI, two forms of the peptides carrying three or four positive charges were mostly detectable, indexes show their corresponding *m/z* values. Large signals at 533.26 (middle panel) and 615.3 (right panel) belong to detergents dodecyl-maltoside (DM) and CHAPS, respectively. (B) Sequence of the synthesized peptide corresponding to the pD1 sequence His337–Gly360 with five histidine residues added in front of the residue His337 forming together hexahistidine tag used for isolation of the peptide. In the position 345 the Ser residue was replaced by Pro to inhibit the final maturation cleavage. Positions of the final cleavage (after residue Ala344) and of the hypothetical primary cleavages (after residues Ala352 and Ala357) are also shown. (C) Analysis of signal $P3_{591}(+4)$ by MS/MS. The peptide HHHHHHNFPLDLAPGEQAPVA was identified by nearly the complete b-ion series.

corresponding to peptide molecules of relative molecular weight 3086 having 3 and 4 positive charges (Fig. 1A and B, P1). These species were also found in the spectrum of peptides isolated after incubation with the cellular extract. However, there were several new dominant peaks and calculation of their mass showed that these were multiply charged forms of two smaller peptides with relative molecular masses 2817 and 2360 (Fig. 1A and B, P2 and P3). The mass of the smaller one corresponded to the original penta-His peptide cut after the residue Ala352 (P3) and its MS/MS spectra after the secondary breakdown of this fragment directly confirmed the sequence identity of the fragment (Fig. 1C). Unexpectedly, there were even more intense signals from the peptide that resulted from cleavage after Ala357 (P2). Thus, the results of the mass spectrometric analysis supported the hypothesis that the intermediate form iD1 could originate from cleavage of pD1 after residue Ala352. However, the results also pointed to the possibility of an additional cleavage step after Ala357.

As we were interested in the identity of the protease(s) responsible for the peptide cleavage, we assessed the formation of peptide fragments in extracts from strains with inactivated CtpA and CtpB maturation proteases. Both smaller peptides (P2 and P3) were generated during incubation with the cellular extract isolated from both the single deletion mutant ΔCtpA and

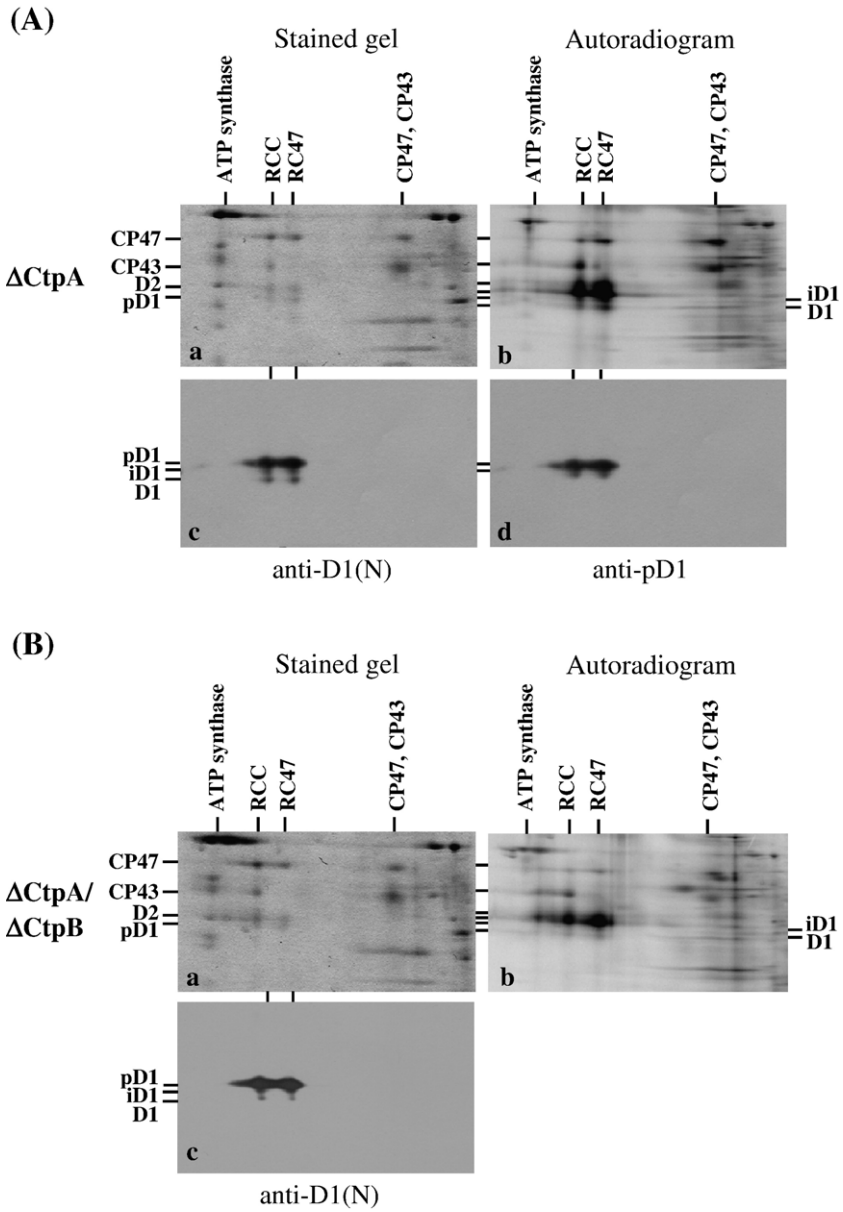

Fig. 2. Coomassie stain, autoradiogram and western blot of thylakoid membrane proteins from the ΔCtpA (A) and ΔCtpA/ΔCtpB (B) strains after separation by 2D PAGE. Radiolabeled thylakoid membrane proteins were separated by a combination of BN-PAGE and SDS-PAGE as described in Materials and methods. Gels were either stained (panels A(a) and B(a)), dried and exposed to film (panels A(b) and B(b)) or electroblotted onto PVDF membrane and immunodecorated using either the antibody raised against the residues 2–17 of the D1 protein (anti-D1(N), panel A(c) and B(c)) or the antibody raised against the C-terminal extension of the precursor form of the D1 protein recognizing iD1 and pD1 forms (anti-pD1, panel A(d)). Loaded thylakoids corresponded to 2 μg of chlorophyll. Designation of complexes: RCC: PSII reaction center core; RC47: PSII reaction center core lacking CP43.

the double deletion mutant ΔCtpA/ ΔCtpB (results not shown). This result showed that the fragments P2 and P3 might result from action of proteases other than CtpA and CtpB. In order to assess cleavage specific just for CtpA, we finally incubated the peptide with purified CtpA protease from *Scenedesmus* which was previously found to act on *Synechocystis* pD1 in vivo [14]. The resulting MS spectrum of peptides (Fig. 1A) showed formation of just the P3 fragment. Thus, P3 appears to be the only true CtpA-specific fragment and consequently iD1 is most probably a form of D1 that is truncated by 8 amino-acid residues leaving Ala352 as the C-terminal residue.

The involvement of proteases other than CtpA in the cleavage of the D1 extension in *Synechocystis* was supported by analysis of radioactively labeled thylakoid proteins from the ΔCtpA mutant by 2D blue-native/SDS gel electrophoresis (BN/SDS-PAGE). In the PSII core and core lacking CP43 (RC47) complexes of the mutant, pD1 and trace amounts of two smaller D1 derivatives ascribed to iD1 and D1 were identified by autoradiography (Fig. 2A,b). In agreement with this designation all three proteins cross-reacted with an N-terminal specific D1 antibody (Fig. 2A,c) while only the upper two reacted with the antibody specific for the C-terminal extension (Fig. 2A,d). When the double mutant lacking CtpA and its homologue CtpB was analyzed by BN/SDS-PAGE, the autoradiogram (Fig. 2B,b) and immunoblots ((Fig. 2B,c) also showed the presence of residual amount of iD1 and D1 but their abundance was lower than in the single mutant ΔCtpA suggesting a certain role of CtpB in this cleavage.

3.2. Characterization of mutants Leu353Pro and Leu353stop supports cleavage of the D1 extension after Ala352

To further support our findings from the in-vitro experiments, we attempted to inhibit the putative cleavage after Ala352 in vivo by replacing the subsequent residue, Leu353, with Pro (Fig. 3A). This experiment was inspired by the previous work of Nixon et al. [5] who found that replacement of Ser residue by Pro at position 345 inhibited the final D1 maturation step after Ala344. The Leu353Pro mutant contained a similar amount of the matured D1 as the control strain A2 showing that the mutation did not inhibit the final cleavage of the extension after Ala344 (Fig. 3B, anti-D1(M)). However, when the strain was incubated under conditions that promote accumulation of iD1 and pD1 in WT (2 h of high irradiance at 20 °C), the D1 extension-specific antibody revealed the increased accumulation of a band that co-migrated with pD1 present in the ΔCtpA strain and, in reduced amounts, also in the control strain A2 (Fig. 3C, anti-pD1). This result supported the hypothesis that the iD1 band does indeed result from cleavage of pD1 after Ala352. There still seemed to be a small amount of the band corresponding to iD1 indicating that a very limited cleavage after Ala352 was still possible in the Leu353Pro mutant. This would be in agreement with data of Hatano-Iwasaki et al. who found that introduction of Pro residue immediately after the D1 maturation site in *Chlamydomonas* does not inhibit the cleavage completely [17]. To obtain further experimental support for inhibition of iD1 formation in the

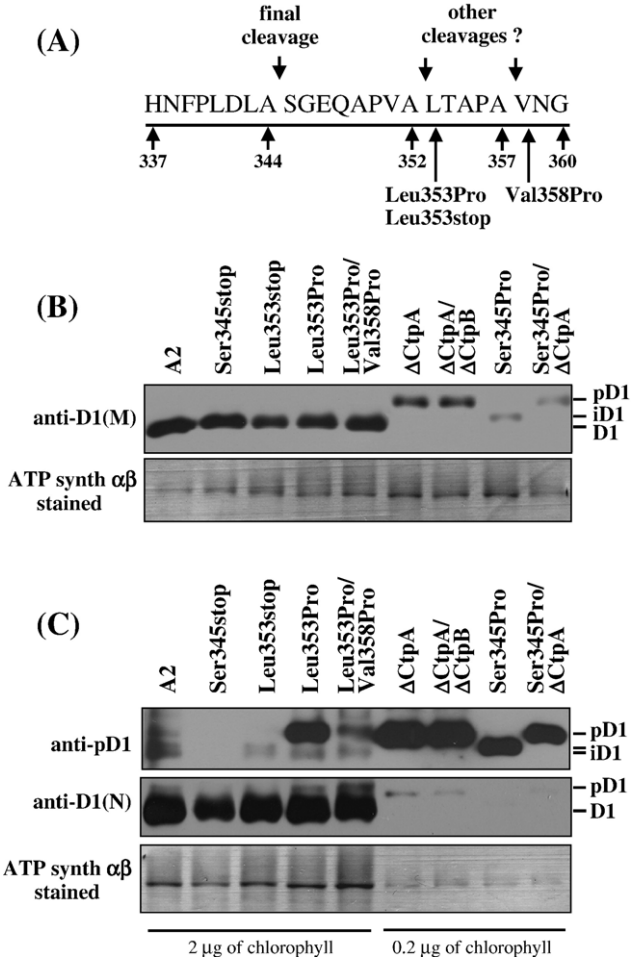

Fig. 3. Amino acid sequence of the C-terminal end of the D1 precursor in the control and mutant strains of *Synechocystis* PCC 6803 (A) and western blot detection of the D1 forms in the cells of ΔCtpA, ΔCtpA/ΔCtpB, Ser345Pro and Ser345Pro/ΔCtpA mutants cultivated under standard conditions (B, C) and cells of the A2, Ser345stop, Leu353stop, Leu353Pro and Leu353Pro/Val358Pro cultivated either under standard conditions (B) or under increased irradiance at low temperature (C). (A) The sequence His337–Gly360 of the C-terminal part of the D1 precursor is shown and mutations in the amino acid residues Leu353 and Val358 are designated by arrows. The final cleavage of the extension after Ala344 and a hypothetical position of other cleavages after Ala352 and Ala357 are also shown. (B, C) Thylakoid membrane proteins from cells of the *Synechocystis* strains A2 and mutants Ser345stop, Leu353stop, Leu353Pro, Leu353Pro/Val358Pro, ΔCtpA, ΔCtpA/ΔCtpB, Ser345Pro and Ser345Pro/ΔCtpA were separated by SDS-PAGE, electroblotted onto PVDF membrane and immunodecorated using: (B) the antibody raised against the residues 59–76 of the D1 protein (anti-D1(M)) recognizing all D1 forms, thylakoids containing 1 μg of chlorophyll and isolated from the standard cultures were loaded for all strains; (C) (i) the antibody raised against the C-terminal extension of the precursor form of the D1 protein (anti-pD1) recognizing iD1 and pD1 forms, or (ii) the antibody raised against the residues 2–17 of the D1 protein (anti-D1(N)) recognizing all D1 forms; loaded thylakoid proteins from A2, Ser345stop, Leu353stop, Leu353Pro and Leu353Pro/Val358Pro strains corresponding to 2 μg of chlorophyll were isolated from cultures that were incubated for 2 h under irradiance of 500 μmol photons m^{-2} s^{-1} at 20 °C; loaded thylakoid proteins from ΔCtpA, ΔCtpA/ΔCtpB, Ser345Pro and Ser345Pro/ΔCtpA strains corresponding to 0.2 μg of chlorophyll were isolated from standard photoheterotrophic cultures. ATP synth αβ stained indicates band of α and β subunit of ATP synthase stained on the blots by Poinceau S to document loading of the gels.

Leu353Pro mutant, we radioactively labeled the mutant cells of the control strain and the Leu353Pro mutant at 20 °C and analyzed thylakoids by 2D BN/SDS-PAGE (Fig. 4A). While in the control strain all three labeled D1 forms were detected, the Leu353Pro contained increased amount of labeled pD1 together with matured D1 but no iD1. Also, pD1 in cells of Leu353Pro labeled at 15 °C was directly converted into matured D1 without any intermediate when the cells were transferred to 30 °C in the presence of chloramphenicol (Fig. 4B). Under the same conditions, the maturation in the wild-type strain clearly proceeds via the intermediary form iD1 [7].

To test further the idea that cleavage occurred after Ala352, a mutant in which the codon for Leu353 of D1 was replaced by the stop codon was analyzed (Fig. 3A). Again, the strain exhibited WT levels of the mature D1 form (Fig. 3B, anti-D1 (M)). This showed that the second C-terminal half of the extension was not required for the final cleavage step. At low temperature the D1 species detected by the extension-specific antibody in the Leu353stop mutant had a similar mobility to that of iD1, again consistent with cleavage after Ala352. No band corresponding to pD1 was found even after incubation of the cells at 20 °C (Fig. 3C, anti-pD1). In the strain Ser345stop lacking the C-terminal extension [18] no bands of iD1 or pD1 were detected confirming the specificity of the antibody (Fig. 3C, anti-pD1).

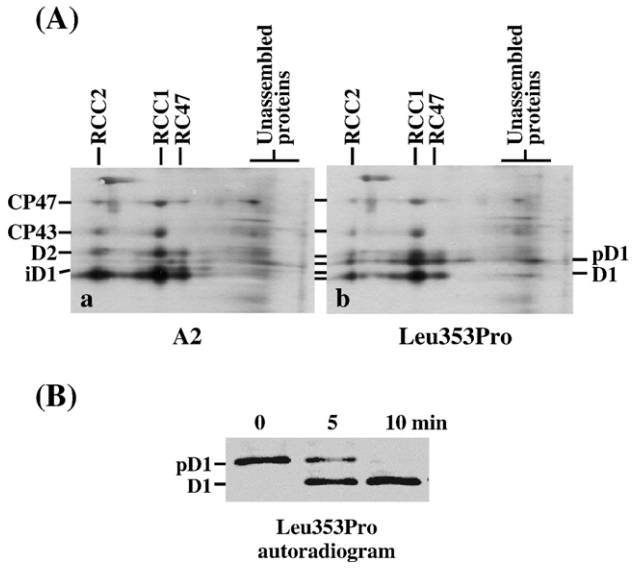

Fig. 4. Autoradiogram after 2D analysis of thylakoid membrane proteins from the strains A2 and Leu353Pro labeled at 20 °C (A) and kinetics of maturation of radioactive pD1 in the Leu353Pro mutant labeled at 15 °C (B). (A) Thylakoid membrane proteins isolated from cells of A2 (a) and Leu353Pro (b) labeled under irradiance of 500 μmol photons. m^{-2} s^{-1} at 20 °C were separated by a combination of BN-PAGE and SDS-PAGE as described in Materials and methods. Gel was stained, dried and exposed to film. (B) Cells of the Leu353Pro mutant were labeled under irradiance of 500 μmol photons m^{-2} s^{-1} at 15 °C for 30 min and after addition of chloramphenicol (1 mg ml^{-1}) the cells were exposed to 125 μmol photons m^{-2} s^{-1} at 30 °C for 0, 5 and 10 min. Thylakoid proteins isolated from the labeled cells were analyzed by 1D SDS-PAGE, electroblotted onto PVDF membrane and exposed to film. Thylakoid proteins corresponding to 2 μg of chlorophyll were loaded per lane. RCC1 and RCC2 are monomeric and dimeric PSII reaction center cores.

3.3. The strain containing proline residues at positions 353 and 358 is also able to mature D1

In order to test the possibility of a cleavage site after Ala357 in vivo, we also constructed and characterized the double mutant Leu353Pro/Val358Pro. Similar to the other strains with point mutations made in the second half of the extension, this mutant was able to mature D1 (Fig. 3B, anti-D1(M)). We checked the accumulation of the unprocessed forms of D1 at low temperature using the anti-extension antibody and found somewhat increased amount of pD1 when compared with the control strain A2 (Fig. 3C, anti-pD1). On the other hand, the amount of pD1 was lower than in the single mutant Leu353Pro, but this could be related to the poorer reactivity of the antibody with the extension containing two exchanged amino-acid residues. This suggestion was supported by immunoblots using the antibody against the N-terminus (Fig. 3C, anti-D1 (N)). The blots also showed that the pD1 band in the Leu353Pro and to a higher extent in the Leu353Pro/Val358Pro strain had slightly decreased mobility than pD1 in the ΔCtpA strains. We assume that this small difference, which was more apparent using the anti-D1(N) antibody, is most probably related to introducing new Pro residues. In conclusion, replacement of Val358 by the Pro residue in addition to that in the position 353 did not abolish the final cleavage of the extension and the obtained data neither exclude, nor confirm, the existence of the cleavage after Ala 357 residue in vivo.

3.4. The non-photoautotrophic strain Ser345Pro does not accumulate pD1 but iD1

The replacement of D1 residue Ser345 by Pro leads to a non-photoautotrophic phenotype due to an inability to form mature D1 [5]. It has been assumed that the D1 form accumulated in the strain is the D1 precursor pD1. However, our analysis showed that the electrophoretic mobility of the D1 form accumulated in the Ser345Pro strain is similar to that of iD1 found in the control strain A2 and in the Leu353stop strain (Fig. 3B). In contrast, the mobility of the pD1 present in the ΔCtpA strain, lacking the D1 maturation protease, CtpA, was lower (Fig. 3B). This result suggested that the mutation Ser345Pro only inhibited the final cleavage step while the other cleavage step could probably still occur. In order to exclude a possible effect of the Ser345Pro mutation on the conformation and mobility of pD1, we constructed the double mutant carrying the Ser345Pro mutation and inactivated CtpA protease (Ser345Pro/ΔCtpA, Fig. 3B). In this strain, the band corresponding to pD1 of ΔCtpA was detected confirming that the accumulated D1 form in the Ser345Pro mutant is iD1. This result also suggests that CtpA plays a key role in the formation of iD1 in vivo.

3.5. The autotrophic strains with modified extension exhibit increased vulnerability to photoinhibition

Mutations in the D1 extension not inhibiting the final maturation step (Leu353Pro, Leu353stop and Leu353Pro/Val358-Pro) did not affect the phenotype of the strains when compared

with the control strain A2. Photoautotrophic growth of the strains under 25 μmol photons m^{-2} s^{-1} of white light was indistinguishable from the WT control and no differences were found among the strains with regard PSII activity. This was expected since the D1 extension is quickly cleaved and it is not present in fully functional PSII. However, as previously shown for other strains either containing a modified D1 extension or lacking the extension [18], the rate of photoinhibition in vivo was affected in the D1 extension mutants described here. Indeed, the measurement of oxygen-evolving activity during exposure of cells to increased irradiance revealed higher vulnerability to photoinhibition of all three site-directed mutants in comparison with the control strain (Fig. 5). This difference was again visible even in the presence of lincomycin [18] and was most of all pronounced in the strains Leu353stop and Leu353Pro/Val358Pro.

4. Discussion

4.1. Cleavage of the C-terminal extension of D1 in Synechocystis

In principle, C-terminal sequencing of intermediate iD1 could be used to identify the location of the cleavage site in the C-terminal extension of pD1. However, iD1 occurs very transiently in *Synechocystis* thylakoids and can be detected only by radioactive labeling or by immunoblotting [7,13], thus making analysis difficult. Therefore, we decided to use two indirect approaches to obtain information about the putative primary cleavage site of the D1 precursor in *Synechocystis*. The first one exploits the approach developed originally by Satoh and colleagues [19] and is based on the in-vitro proteolysis of a synthetic peptide encompassing the C-terminal region of pD1. The results indicated the peptide bond between Ala352 and Leu353 as the cleavage site. Surprisingly, it appeared that Ala at position 357 might also be a target for proteolytic cleavage by proteases other than CtpA and CtpB. In addition to Ala344, Ala352 and Ala357, the D1 extension in *Synechocystis* contains two other Ala residues at positions 349 and 355. These two residues are followed by Pro and no traces of peptides corresponding to fragmentation in these two positions were identified in the peptide mixture after the in-vitro proteolysis of the original peptide incubated even with the extract from the CtpA/CtpB double mutant. The result suggested that this CtpA-unrelated cleavage of the pD1 extension, which was also confirmed by 2D analysis of radiolabeled proteins, seems to be specific for Ala residues unless they are followed by the Pro residue. The obvious candidate for this proteolytic activity is the Tsp-like carboxyl-terminal protease (CtpC) encoded by the *slr1751* gene which has been localized to both the cytoplasmic membrane and the periplasmic space [20]. Its role could not be directly checked by knock-out of the *slr1751* gene since it cannot be inactivated without loss of cell viability [21]. Nevertheless, the negligible accumulation of mature D1 in the CtpA/CtpB deletion strain shows that pD1 maturation in this strain does not proceed at physiologically significant rates.

The second approach for the determination of the initial cleavage site was based on the construction of mutants with site-specific mutations designed to affect cleavage of pD1 at the sites identified in the in-vitro experiments. By analogy with the inhibition of the CtpA-mediated D1 proteolysis after the Ala344 residue, a Pro residue was introduced into position 353 in an attempt to inhibit cleavage at this site. Indeed, this was documented by the increased accumulation of pD1 at 20 °C. Similarly the absence of pD1 and detection of a D1 species with identical mobility as iD1 in the mutant Leu353stop was again in agreement with the cleavage site at the position Ala352. The good correspondence between the predicted effect of both mutations and the obtained experimental data also reduced the possibility that iD1 is a result of posttranslational modification or conformational change of pD1 or D1 [16]. Nevertheless, careful inspection of iD1 bands in the strains A2, Leu353Pro, Leu353stop and Leu353Pro/Val358Pro revealed that iD1 is present in the form of double band (Fig. 3B) and so we cannot exclude that some additional post-translational modification of iD1 occurs.

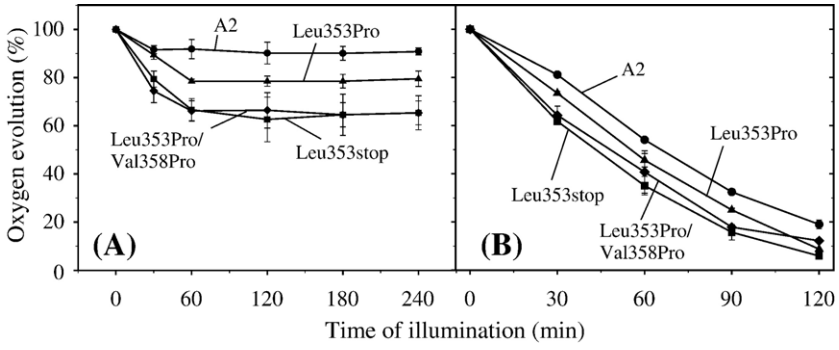

Fig. 5. Time course of PSII oxygen evolution in cells of the *Synechocystis* PCC 6803 strains subjected to high irradiance in the absence (−LIN) or presence (+LIN) of lincomycin. Cells of the A2 (circles), Leu353stop (squares), Leu353Pro (triangles) and Leu353Pro/Val358Pro (diamonds) were illuminated with 500 μmol photons m^{-2} s^{-1} of white light for 480 min in the absence (left panel) or for 120 min in the presence (right panel) of 100 μg ml^{-1} lincomycin. Aliquots of the suspensions were taken during illumination at the times indicated and oxygen evolving activity in the presence of the artificial electron acceptors (HRA) was assayed in whole cells as described in Materials and methods. Values in the plot represent mean of 3 measurements. Initial values were in the range 550–660 μmol O_2 mg Chl^{-1} h^{-1}.

4.2. Physiological importance of iD1 and the role of the CtpB protease

Formation of the D1 maturation intermediate iD1 in the cyanobacterium *Synechocystis* may be closely related to the length of its D1 extension which is about twice as long as the one in higher plants. The reason for the longer extension and its multiple cleavage may reflect specific features of PSII biogenesis in cyanobacteria. There are indications that in cyanobacteria de-novo assembly of PSII occurs in the cytoplasmic membrane [22] while in higher plants this role is most probably played by stromal lamellae [23]. In the early stages of the cyanobacterial PSII assembly the reaction center complex (RC) is formed consisting of D2, cytochrome b-559 and either intermediate or mature D1 [13]. The presence of iD1 is a typical feature of the RC and may reflect a specific localization of the complex, for instance in the cytoplasmic membrane. The presence of the partially matured D1 could be an important structural determinant that optimizes the targeting of the RC into thylakoids where attachment of CP47 and the final D1 maturation step occurs. It is typical that in the PSII core complex lacking CP43 the D1 protein is almost exclusively found in the mature form [13]. The absence of iD1 suggests that in this complex pD1 is directly cleaved at Ala344 as in the strain Leu353Pro or that iD1 is rapidly converted to mature D1.

We have also attempted to establish a possible role for the CtpB protease in D1 maturation. It is evident that the amount of the D1 precursor is very similar in the single ΔCtpA and the double ΔCtpA/ΔCtpB mutants. This result agrees with the previous characterization of the mutants [21]. Nevertheless, we cannot exclude some minor role of CtpB in D1 maturation as the trace amount of iD1 and D1 was higher in the single ΔCtpA mutant than in the double ΔCtpA/ΔCtpB mutant. CtpB could, for instance, perform the complete proteolytic degradation of the cleaved extension in the periplasmic space in which the protease was identified [24].

4.3. Maturation as a possible rate limiting factor in PSII repair in the autotrophic strains with modified D1 extension

Recently, we have shown that the mutant lacking the entire pD1 extension or mutants with the residue Asn359 replaced either by Asp or by His [18] exhibited increased vulnerability to photoinhibition when compared with the control stain having the original extension. The importance of the native D1 extension for the optimal photosynthetic performance of *Synechocystis* under increased irradiance has been already established [25] and has been again confirmed by the characterization of the light-sensitivity of Photosystem II in the new site-directed mutants Leu353Pro, Leu353stop and Leu353Pro/Val358Pro. When compared with the control strain, A2, PSII activity declined to a greater degree in all three mutants. We assume that the light sensitivity of Leu353Pro and Leu353Pro/Val358Pro is caused by a slowed-down maturation (Fig. 3C) that might become the rate-limiting step in the PSII repair. Interestingly, the mutant Leu353stop seems to mature iD1 faster than strain Leu353Pro matures pD1 (judged from the low level of iD1 in Leu353stop and high level of pD1 in Leu353Pro) but it is more sensitive to photoinhibition. It is difficult to explain this result but we might speculate that the last 8 residues of pD1 missing in the Leu353stop mutant plays an important role for the fitness of the cyanobacterium under high light conditions, such as for instance by acting as a signaling molecule (see [18]).

In agreement with previous results [18], the difference in the sensitivity to photoinhibition among the strains was also apparent in the presence a protein synthesis inhibitor lincomycin. As mentioned above, we assume that maturation of pD1 is slowed down due to modification of the extension and somewhat limits the efficiency of the PSII repair cycle. The cytoplasmic membrane is proposed to be the site of D1 maturation and is the membrane layer most exposed to radiation since the thylakoid layers localized inside the cell may shade each other. It is possible that slower maturation of D1 causes increased retention of PSII complexes in the membrane layers close to the surface which may lead to higher rates of photoinhibition even in the presence of lincomycin. This might be an additional explanation to those provided in [18] for the higher susceptibility of the D1 extension mutants in the presence of protein synthesis inhibitors.

Acknowledgements

The authors are grateful to Dr. Dexter A. Chisholm for constructing the ΔCtpA and ΔCtpA/ΔCtpB strains, to Remco De Vries for construction of the S345P/ΔCtpA mutant and to Prof. E.-M Aro for the kind gift of the antiserum specific for the first 15 amino-acid residues of D1 from *Synechocystis*. The work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (project no. MSM6007665808), by the Czech Academy of Sciences (Institutional Research Concept no. AV0Z50200510) and by the BBSRC.

References

[1] A.K. Mattoo, J.B. Marder, M. Edelman, Dynamics of the photosystem II reaction center, Cell 56 (1989) 241–246.
[2] C.H. Jansson, R.J. Debus, H.D. Osiewacz, M. Gurevitz, L. Mc Intosh, Construction of an obligate photoheterotrophic mutant of the cyanobacterium *Synechocystis* 6803, Plant Physiol. 85 (1987) 1021–1025.
[3] L. Zhang, E.-M. Aro, Synthesis membrane insertion and assembly of the chloroplast-encoded D1 protein into photosystem II, FEBS Lett. 512 (2002) 13–18.
[4] P.R. Anbudurai, T.S. Mor, I. Ohad, S.V. Shestakov, H.B. Pakrasi, The CtpA gene encodes the C-terminal processing protease for the D1 protein of the photosystem-II reaction-center complex, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 8082–8086.
[5] P.J. Nixon, J.T. Trost, B.A. Diner, Role of the carboxy terminus of polypeptide-D1 in the assembly of a functional water-oxidizing manganese cluster in Photosystem-II of the cyanobacterium *Synechocystis* sp. PCC 6803-assembly requires a free carboxyl group at C-terminal position 344, Biochemistry 31 (1992) 10859–10871.
[6] J.L. Roose, H.B. Pakrasi, Evidence that D1 processing is required for manganese binding and extrinsic protein assembly into photosystem II, J. Biol. Chem. 279 (2004) 45417–45422.
[7] N. Inagaki, Y. Yamamoto, K. Satoh, A sequential two-step proteolytic process in the carboxyl-terminal truncation of precursor D1 protein in *Synechocystis* sp. PCC 6803, FEBS Lett. 509 (2001) 197–201.

[8] J.G.K. Williams, Construction of specific mutations in PSII photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803, Methods Enzymol. 167 (1998) 766–778.
[9] M. Tichý, L. Lupínková, C. Sicora, I. Vass, S. Kuviková, O. Prášil, J. Komenda, Synechocystis 6803 mutants expressing distinct forms of the Photosystem II D1 protein from *Synechococcus* 7942: relationship between the psbA coding region and sensitivity to visible and UV-B radiation, Biochim. Biophys. Acta 1605 (2003) 55–66.
[10] H.B. Pakrasi, J.G.K. Williams, C.J. Arntzen, Targeted mutagenesis of the psbE and psbF blocks photosynthetic electron transport: evidence for a functional role of cytochrome b-559 in photosystem II, EMBO J. 7 (1988) 325–332.
[11] S.H. Ke, E.L. Madison, Rapid and efficient site-directed mutagenesis by single-tube 'megaprimer' PCR method, Nucleic Acids Res. 25 (1997) 3371–3372.
[12] J. Komenda, L. Lupínková, J. Kopecký, Absence of the *psbH* gene product destabilizes photosystem II complex and bicarbonate binding on its acceptor side in *Synechocystis* PCC 6803, Eur. J. Biochem. 269 (2002) 610–619.
[13] J. Komenda, V. Reisinger, B.Ch. Müller, M. Dobáková, B. Granvogl, L.A. Eichacker, Accumulation of the D2 protein is a key regulatory step for assembly of the photosystem II reaction center complex in *Synechocystis* PCC 6803, J. Biol. Chem. 279 (2004) 48620–48629.
[14] D.I. Liao, J. Qian, D.A. Chisholm, D.B. Jordan, B.A. Diner, Crystal structures of the photosystem II D1 C-terminal processing protease, Nat. Struct. Biol. 7 (2000) 749–753.
[15] A.R. Wellburn, K. Lichtenthaler, Formulae and programs to determine total carotenoids and chlorophyll a and b of leaf extracts in different solvents, in: C. Sybesma (Ed.), Advances In Photosynthesis Research, Martinus Nijjhoff, Dordrecht, 1984, pp. 10–12.
[16] T. Jansen, E. Kanervo, E.-M. Aro, P. Maenpaa, Localisation and processing of the precursor form of photosystem II protein D1 in *Synechocystis* 6803, J. Plant Physiol. 159 (2002) 1205–1211.
[17] A. Hatano-Iwasaki, J. Minagawa, Y. Inoue, Y. Takahashi, Characterization of chloroplast psbA transformants of *Chlamydomonas reinhardtii* with impaired processing of a precursor of a photosystem II reaction center protein, D1, Plant Mol. Biol. 42 (2000) 353–363.
[18] S. Kuviková, M. Tichý, J. Komenda, A role of the C-terminal extension of the Photosystem II D1 protein in sensitivity of the cyanobacterium *Synechocystis* PCC 6803 to photoinhibition, Photochem. Photobiol. Sci. 4 (2005) 1044–1048.
[19] F. Taguchi, Y. Yamamoto, N. Inagaki, K. Satoh, Recognition signal for the C-terminal processing protease of D 1 precursor protein in the photosystem II reaction center, an analysis using synthetic oligopeptides, FEBS Lett. 512 (1989) 227–231.
[20] F. Huang, A. Persson, H.B. Pakrasi, B. Andersson, B. Norling, Proteomics of *Synechocystis* sp. Strain PCC 6803. Identification of plasma membrane proteins, Mol. Cell. Proteomics 1 (2002) 956–966.
[21] N.B. Ivleva, K.V. Sidoruk, H.B. Pakrasi, S.V. Shestakov, Investigation of the functional role of Ctp proteins in the cyanobacterium *Synechocystis* sp. PCC 6803, Microbiology (Rus) 71 (2002) 509–513.
[22] E. Zak, B. Norling, R. Maitra, F. Huang, B. Andersson, H.B. Pakrasi, The initial steps of biogenesis of cyanobacterial photosystems occur in plasma membranes, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 13443–13448.
[23] F.E. Callahan, W.P. Wergin, N. Nelson, M. Edelman, A.K. Mattoo, Distribution of thylakoid proteins between stromal and granal lamellae in *Spirodela* — dual location of Photosystem-II components, Plant Physiol. 91 (1989) 629–635.
[24] S. Fulda, F. Huang, F. Nilsson, M. Hagemann, B. Norling, Proteomics of *Synechocystis* sp. strain PCC 6803. Identification of periplasmic proteins in cells grown at low and high salt concentrations, Eur. J. Biochem. 267 (2000) 5900–5907.
[25] N.B. Ivleva, S.V. Shestakov, H.B. Pakrasi, The carboxyl-terminal extension of the precursor D1 protein of Photosystem II is required for optimal photosynthetic performance of the cyanobacterium *Synechocystis* sp. PCC 6803, Plant Physiol. 124 (2000) 1403–1411.