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Mutagenesis of the D-E loop of photosystem II reaction centre protein D1. Function and assembly of photosystem II

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Abstract

The sequence connecting α-helices D and E of the D1 protein in photosystem II (PSII) is longer than that found in the corresponding loop of the L subunit in the rhodobacterial reaction centre. This sequence was mutated in order to determine its role in oxygenic photosynthesis. Site-specific mutants, including point mutations and deletions of different size, of the PEST-like region and the putative cleavage area in the D-E loop of the D1 protein were constructed in *Synechocystis* sp. PCC 6803. The effects of mutations on the functional and structural properties of PSII and turnover of the D1 protein were examined. Our results demonstrate that deletion of either the PEST-like sequence (ΔR225-F239) or the putative cleavage region (ΔG240-V249, ΔR225-V249) of the D1 protein resulted in severe perturbations on the function of the Q_B electron acceptor of PSII. However, PSII centres of the mutant with deleted PEST region remained functional enough to support autotrophic growth whereas deletions of the putative cleavage region prevented autotrophic growth. Although enhanced degradation rates of the mutant D1 proteins under low-light growth conditions demonstrate that neither the PEST-like sequence nor the putative cleavage region are required for D1 proteolysis, it became clear that the extension in the D-E loop of the D1 protein is essential for proper PSII assembly and photoautotrophic growth. Moreover, modifications of the D-E loop resulted in transcriptional activation of the *psbA* gene, indicating that neither light intensity, as such, nor the activity of the electron transfer chain are the only determinants in regulation of *psbA* gene transcription.

Introduction

Photosystem II performs the essential reaction of oxidation of water and reduction of plastoquinone (PQ) in photosynthesis of plants, algae and cyanobacteria. The heart of PSII consists primarily of the D1 and D2 reaction centre proteins encoded by the *psbA* and *psbD* genes, respectively. The D1-D2 heterodimer is postulated to harbour all the redox components involved in the electron transfer reactions of PSII from water to the second plastoquinone electron acceptor Q_B [26, 43], and the functional form of this enzyme complex is thought to be a dimer [2, 3]. The D1 and D2 proteins are composed of five membrane spanning α-helices [36, 43] as predicted from comparison of the amino acid sequences and hydropathy index plots of the D1 protein with the folding of the L subunit of rhodobacterial non-oxygenic photosynthetic reaction centre, whose 3-dimensional structure has been resolved [9].

Comparison with the L subunit of the rhodobacterial reaction centre [47, 54] revealed longer A-B and D-E loops and a longer carboxy-terminal extension in the D1 protein than in the L subunit. The stromal/cytosolic loop of the D1 protein connecting helices D and E has been shown to have a crucial role in PSII electron transfer, as amino acids of the loop affect the binding and function of Q_B and the non-heme iron of PSII [10, 43]. The D1 protein also exhibits a higher rate of light-dependent turnover *in vivo* [21] than any other thylakoid protein; the rapid turnover is probably related to strong oxidants produced by PSII. The L subunit does not undergo such a rapid light-dependent

turnover. The extended D-E loop together with the appearance of a PEST-like sequence in the loop (see below) might thus be involved in the rapid turnover of the D1 protein. The extended amino acid stretch in the D-E loop of the D1 protein has been divided into two functional domains: the PEST-like sequence and a putative cleavage area [13]. The PEST-like sequence is rich in glutamate (E), serine (S) and threonine (T) but contains no proline (P) residues that are usually characteristic of PEST sequences found in other proteins having a high turnover rate (for a review, see [32]). The primary cleavage site of the D1 protein is thought to map in the D-E loop either between amino acid residues 238 and 248 or more precisely inside or in close proximity to the QEEET motif between amino acids 241–245 [13, 39, 44]. Although mutations in the D-E loop of the D1 protein have been shown to affect the functional properties of PSII and the turnover rate of the D1 protein [17, 18, 25, 28–30, 49], neither the properties of PEST-like sequence nor the exact cleavage site have been confirmed. Moreover, these studies have revealed interesting relationships between D1 protein degradation and *psbA* gene transcription and translation pointing to a feedback regulation of *psbA* gene expression in *Synechocystis* sp. PCC 6803 [50].

To determine the role of the longer sequence in the D-E loop of the D1 protein than that found in the L subunit, a series of new site-specific D-E loop mutations of the D1 protein, including the largest deletions of the D-E loop so far reported, were introduced into the *psbA-2* gene of *Synechocystis* sp. PCC 6803. The mutant strains were employed to determine the expression of the *psbA-2* gene (e.g. transcription of the *psbA-2* gene, D1 protein degradation rate and PSII assembly) and electron transfer properties in PSII.

Materials and methods

Bacterial strains and growth conditions

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803, referred here as wild-type (WT), was grown in BG-11 medium [53] under photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1} at 32 °C. Transformation host A2, with *psbA-1* and *psbA-3* genes interrupted by kanamycin and chloramphenicol resistance genes, respectively, was grown as described [23]. The mutagenesis cartridge, containing a streptomycin and spectinomycin resistance cassette, was introduced into the A2 strain without any mutation in the *psbA-2* gene resulting in the AR control strain [25]. Control strain AR and point mutants E231D and E243Q were grown photoautotrophically as described [25]. Agar plates for the PD (ΔR225-F239), CD (ΔG240-V249), PCD (ΔR225-V249) and LC (ΔT227-Y246) deletion mutants were supplemented with the same antibiotics as for AR, and 5 mM glucose and 10 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethyl urea) were added (also to the plates for the autotrophic strain PD) in order to prevent any selective advantage of possible revertants or secondary mutants. Glucose (5 mM) was added to the liquid medium for the deletion mutants CD, PCD and LC, while PD was grown photoautotrophically in liquid medium unless otherwise indicated.

Molecular cloning, site-directed mutagenesis, transformation and DNA analysis

The genomic DNA of *Synechocystis* 6803 was isolated using the procedure outlined by Williams [53] and all cloning procedures were performed using standard methods [34]. Site-specific *in vitro* mutagenesis was perfomed according to [19], and the oligonucleotides used are presented in Table 1. The vectors and procedures for mutagenesis and transformation are described in detail elsewhere [25]. All the mutations were introduced into the *psbA-2* gene, which is the only active *psbA* gene in *Synechocystis* 6803 strain A2 [14]. The transformation of the strain A2 was performed as described [53] and the selection of the mutated cells as described [25]. After selection, genomic DNA of the transformants was extracted [53], 1.9 kb *Hin*dIII fragment containing the *psbA-2* gene [25] was isolated and used as a template for PCR. The sense strand of the double-stranded PCR product was thereafter sequenced using the procedure of [7] in order to verify that only the mutations constructed were present in the *psbA-2* gene.

Extraction of RNA and northern blotting

Synechocystis cells were harvested at the logarithmic growth stage by centrifugation (4000 $\times$ *g*, 10 min), and RNA was isolated using the hot phenol method in the presence of 60 mM EDTA and 0.5% SDS [37]. Northern blotting was performed using standard methods [34] as described in detail [50]. The coding region of the *psbA-2* gene (amplified with PCR) and the *psbD* gene (from plasmid pRD655) of *Synechocystis* sp. PCC 6803 [8] were used as probes. Autoradiograms were

Table 1. Oligonucleotides used in site-specific mutagenesis. Mutated nucleotides are denoted with a star (∗) and deletions with *(del)*. For designation of the mutant strains, see Fig. 1.

Mutant	Oligonucleotide	Length (bp)
PD	5′-TTCCTTGGTAACCTCCTCCTTGGTG(del)GGTCAAGAAGAAGAAACCTACAACA-3′	50
CD	5′-CAGAACTACGGTTACAAATTC(del)GCCGCCCACGGCTACTTTGGT-3′	42
PCD	5′-TTCCTTGGTAACCTCCTCCTTGGTG(del)GCCGCCCACGGCTACTTTGGTCGGT-3′	50
LC	5′-GGTAACCTCCTCCTTGGTGCGTGAA(del)AACATCGTTGCCGCCCACGGCTACT-3′	50
E231D	5′-ACCGAAGTTGAC∗TCCCAGAACTAC-3′	24
E243Q	5′-TACAAATTCGGTCAAGAAC∗AAGAAACC-3′	27

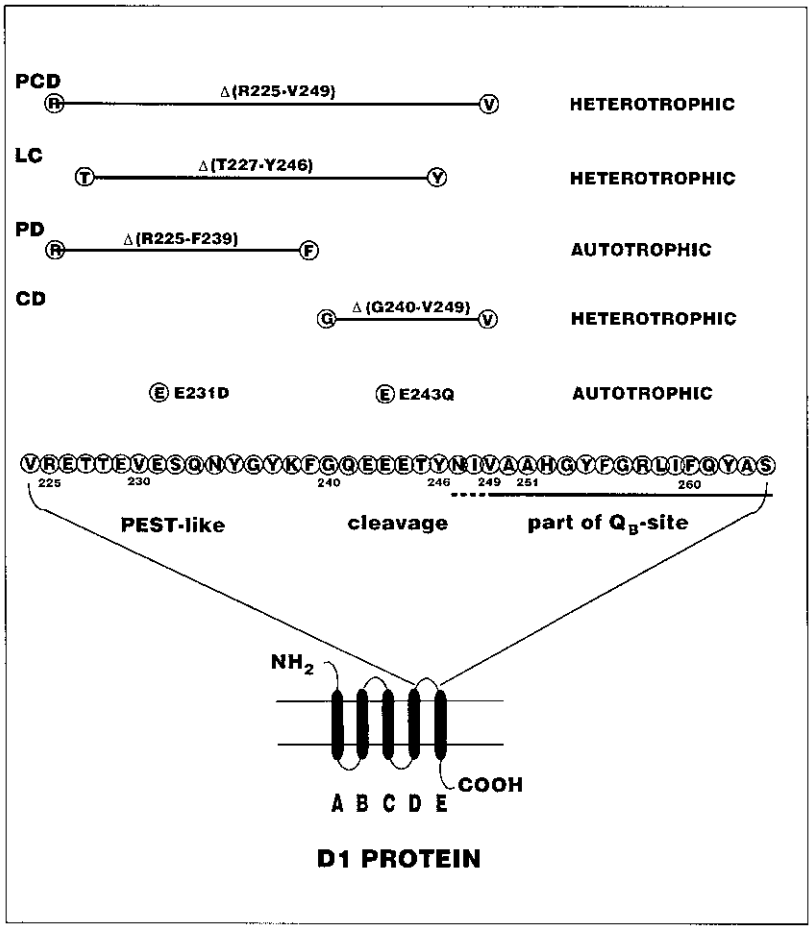

Figure 1. New site-specific mutations in the D-E loop of the D1 protein in *Synechocystis* sp. PCC 6803. Wild-type amino acid sequence of the D-E loop, and the location of the PEST-like sequence, the putative cleavage region, amino acids involved in Q_B site activities in the D-E loop and the new mutations are indicated. The growth mode of mutant strains is also indicated.

scanned with a laser densitometer (LKB). To verify equal loading of the wells and even transfer of the RNA in blotting, all membranes were reprobed with the PstI fragment of the pAN4 vector containing *rrn* genes of *Anacystis nidulans* [42]. Plasmids pRD655 and pAN4 were kindly provided by Dr Christer Jansson and Dr Jonas Lidholm, respectively.

To measure the *psbA* transcript half-life, the *Synechocystis* cell culture (10 μg chlorophyll (Chl)/ml) was incubated under a PPFD of 3 or 1500 μmol m^{-2} s^{-1}, as indicated, at 32 °C in the presence of rifampicin (500 μg/ml), an inhibitor of transcriptional initiation. Aliquots for RNA isolation and northern blotting were taken after 0, 10, 20 and 30 min of incubation and immediately frozen in liquid nitrogen.

Gel electrophoresis and immunological techniques

SDS-page

Photosynthetic membranes of *Synechocystis* were isolated and polypeptides separated as described earlier [49] using 12% SDS- polyacrylamide gels including 4 M urea [20]. Solubilized membranes containing 1 μg Chl (western blots) or 2 μg Chl (pulse-chase) were loaded into each well.

Deriphat page

Thylakoid samples (0.5 mg Chl/ml) isolated using the method of Gombas *et al.* [11] were first solubilized in 2% dodecyl-β-D-maltoside on ice for 7 min and non-denaturating Deriphat (lauryl β-D-iminopropionidate) PAGE of the chlorophyll protein complexes was performed on 5–16% acrylamide gradient gels as originally described [31] with the modifications of [2]. Fifteen μg chlorophyll was loaded into each well.

Immunoblotting

After electrophoresis, the polypeptides or chlorophyll protein complexes were electrotransferred to a polyvinylidene fluoride (PVDF) membrane. Membranes containing radioactive samples were first exposed to X-ray films, and the membrane polypeptides subsequently immunodetected using a chemiluminescence kit (BioRad) and quantified with a laser densitometer (LKB). Two different commercial D1 antibodies (Research Genetics, Alabama) were used; one was raised against the N-terminal amino acid sequence 2–17 (TTTLQQRESASLWEQF) and the other against the D-E loop amino acids 234–242 (NYGYKFGQE) of *Synechocystis* 6803 D1 protein. The commercial D2 antibody (Research Genetics, Alabama) was raised against amino acid sequence 230–245 (NTFRAFEPTQAEETYS) of *Synechocystis* 6803 D2 protein. Polyclonal antibodies against 43 and 47 kDa Chl *a* binding proteins, the antibody against α-subunit of cytochrome b_{559} (cyt b_{559}) and the 33 kDa protein of the oxygen-evolving complex were kind gifts from Professor B. Andersson, Dr R. Barbato and Professor M. Ikeuchi.

Determination of D1-protein degradation

In vivo pulse and chase experiments of thylakoid proteins were performed as described [49]. The cell suspension containing 10 μg Chl/ml was supplemented with ^{35}S-methionine (1000 Ci/mmol) at a final concentration of 1.2 μM and the cells were pulse-labelled for 45 min under growth conditions. Unlabelled methionine (1 mM) was added, and the cells washed and subsequently resuspended in fresh BG-11 medium supplemented with 1 mM cold methionine. Radioactivity was chased for 0, 1, 2 and 3 h under growth light at 32 °C. Autoradiograms were quantified with a laser densitometer and half-lives of the D1 protein were determined by fitting the data to a first-order equation.

Occasionally, the degradation of the D1 protein under growth-light conditions was followed by immunoblotting. Lincomycin (500 μg/ml), an inhibitor of translation initiation was added to the cell suspension containing 10 μg Chl/ml. During the 24 h incubation, 10-ml samples were withdrawn every third hour for isolation of the thylakoid membranes. Membrane polypeptides were separated by SDS-PAGE, and the D1-protein content analysed by immunoblotting.

Oxygen evolution measurements in vivo

For each measurement, an aliquot of cell culture containing 10 μg chlorophyll was spun down and resuspended in 1 ml of fresh BG-11 medium. Oxygen evolution was measured under saturating white light with a Clark-type oxygen electrode (Hansatech) at 32 °C. Either 0.25 mM DCBQ (2,6-dichloro-*p*-benzoquinone) or 0.25 mM DMBQ (2,5-dimethyl-*p*-benzoquinone) was used as the electron acceptor, and 0.25 mM ferricyanide added to keep the quinones in an oxidized form.

Chlorophyll fluorescence

AR and mutant cells were suspended in BG-11 medium at a chlorophyll concentration of 10 μg/ml and the decay of chlorophyll *a* fluorescence yield after a single turnover flash was measured at 20 °C with a PAM fluorometer (Walz, Effeltrich, Germany). A xenon discharge lamp (XST-103) with flash duration of a few microseconds was used for excitation. Fluorescence was probed with a blue (450 nm) measuring beam and detected at right angle through a Schott RG 9 red filter. The rapid phase of the fluorescence relaxation was sampled at 57 kHz for 15 ms, and 50 curves were recorded at 5 s intervals and averaged. The measuring beam was switched off during each dark interval. The fluorometer was remote controlled and the data were analyzed with the FIP fluorescence software (Q_A-Data, Turku, Finland).

Chlorophyll determination

Chlorophyll *a* was determined from cell suspensions as described [4] and from isolated membranes as described [1].

Results

Constructed mutant strains

Different domains of the D-E loop of the D1 protein were deleted in *Synechocystis* sp. PCC 6803 (Fig. 1). In the mutant designated PD (**P**EST region **D**eletion; ΔR225-F239), the amino acid sequence roughly corresponding the PEST-like sequence of the D1 protein [13] was deleted. In the strain designated CD (**C**levage region **D**eletion; ΔG240-V249), the deleted region consisted of amino acids earlier proposed to be essential in the primary cleavage of the D1 protein [13, 39]. To make the mutated D1 protein more closely resemble the L subunit of rhodobacterial reaction centre [41, 45, 47, 54], the PEST-like and cleavage regions were simultaneously deleted to yield the PCD strain (**P**EST/**C**leavage region **D**eletion; ΔR225-V249) and another strain with a slightly shorter deletion designated LC (**L** **C**omparison; ΔT227-Y246). We have also constructed two new point mutations: E231D to dilute the possible PEST property, and E243Q to disrupt the sequence of the three negative charges (E242-E244) in the putative cleavage region. The entire *psbA-2* gene including the untranslated upstream region of the *psbA-2* gene up to –49, the starting site of transcription [22], of the mutant strains was sequenced. The site-directed mutations were present (Fig. 2) and no other mutations were found.

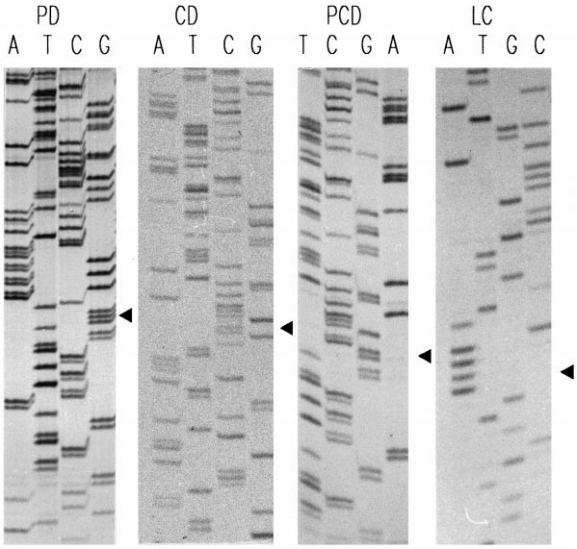

Figure 2. Sequencing autoradiograms presenting the site-specific deletion mutations introduced into the *psbA-2* gene of *Synechocystis* 6803. Arrowheads indicate the sites of deletions. Specifications of the mutant strains PD, CD, PCD and LC are presented in Fig. 1.

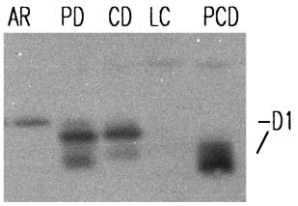

Figure 3. Modified electrophoretic mobility and the content of the D1 protein in *Synechocystis* 6803 deletion mutants under growth conditions as demonstrated by immunoblotting with N-terminal D1-protein antibody. The lower band found in the deletion mutants might represent a conformer form of the mutant D1 protein.

The point mutants E231D and E243Q and the deletion mutant PD were able to grow autotrophically, while the other deletion mutants CD, PCD and LC had lost their capacity for autotrophic growth.

PSII core polypeptides in the mutant strains

Thylakoid membranes of the PD, CD and PCD strains contained elevated amounts of the D1 protein versus chlorophyll when compared to the control strain AR (Fig. 3). Even loading of the gels (1 μg Chl) and the similar overall protein content of the membranes of

Table 2. Relative content of PSII polypeptides in the *Synechocystis* 6803 site-specific D1 mutants. Amounts of polypeptides estimated from three to four immunoblots are normalized to those in the AR control strain. For designation of the mutant strains, see Fig. 1.

Strain	PSII polypeptides					
	D1	D2	43 kDa	47 kDa	cyt b_{559}	33 kDa
AR	1	1	1	1	1	1
PD	3.3	3.8	3.1	3.8	2.0	1
CD	2.8	2.6	3.0	1.9	1.2	1
PCD	3.8	3.4	2.9	2.1	1.7	3.3
LC	tr.[1]	tr.	1.3	tr.	1.7	3.9
E231D	1	0.7	nd[2]	nd	0.8	nd
E243Q	0.8	1.4	nd	nd	1	nd

[1]tr., traces; [2]nd, not determined

different strains were verified by staining the gels with Coomassie brilliant blue (data not shown). The mutated D1 proteins of the PD, CD and PCD strains exhibited faster electrophoretic mobility than did the native D1 protein, with the shift in migration depending on the size of the deletion. The D1 protein of the mutants with deletions in the D-E loop could only be immunodetected using the antibody raised against the N-terminal part of the D1 protein (Fig. 3), whereas the D-E loop antibody could not recognize D1 proteins of these mutants (data not shown) and was therefore regarded as a criterion for the purity of these strains throughout the experiments. The membranes of the PD, CD and PCD strains also contained higher amounts of the D2 protein, cytochrome b_{559}, 43 kDa and 47 kDa Chl *a* binding proteins than did the membranes of the AR control strain (Table 2). The levels of PSII polypeptides in the E231D and E243Q point mutants remained essentially unchanged, though the electrophoretic mobility of the D1 protein of the strain E243Q was somewhat faster than that of the AR control strain (data not shown).

Unlike the other mutants, only traces of D1 protein could be immunodetected from the membranes of the LC strain (Fig. 3). To determine whether stable accumulation of the D1 protein might be affected by different light conditions, LC cells were either incubated for three days in the dark or at PPFD levels of 3, 40 or 150 μmol m^{-2} s^{-1}, but no detectable amounts of the D1 protein could be found in the membranes of

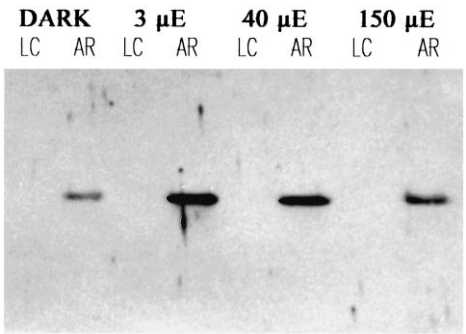

Figure 4. Immunological determination of the D1 protein in the *Synechocystis* 6803 deletion mutant LC and control strain AR after 3 days of growth in the dark and under photosynthetic photon flux density of 3, 40 or 150 μmol m^{-2} s^{-1}.

LC under any of these conditions (Fig. 4). The absence of the D1 protein in LC was also reflected in some other PSII polypeptides, with only traces of D2 protein and 47 kDa Chl *a* binding protein being detected in the membranes of strain LC. However, the 43 kDa protein and cyt b_{559} were abundant in the membranes of LC. The extrinsic 33 kDa polypeptide was found to be present in all the deletion mutants, including LC (Table 2), with even higher amounts being detected in the membranes of PCD and LC than in those of AR.

To determine whether PSII centres with mutated D1 protein exist as dimers or monomers, non-denaturating Deriphat PAGE of the pigment-protein complexes was

performed, and D1 protein immunodetected. Figure 5 shows that D1 protein of PD, CD and PCD strains could be detected in both the PSII monomers and dimers, at higher levels than found in the control strain.

Oxygen evolution and chlorophyll a *fluorescence relaxation*

Since part of the D-E loop is known to interact with the second plastoquinone electron acceptor of PSII, Q_B, the electron transfer properties at the Q_B site were studied in the mutants. The Q_B site provides the Q_B molecule with a suitable position for electron transfer from Q_A to Q_B/Q_B^-, and also allows the rapid exchange of quinol from the Q_B site to the plastoquinone pool (exchange reaction). We used oxygen evolution with DCBQ and DMBQ as artificial electron acceptors to distinguish between effects of the mutations on electron transfer between Q_A and Q_B and the exchange reaction. DCBQ is believed to bind more efficiently to the Q_B niche than DMBQ; thus the main electron accepting site of DMBQ is the reduced plastoquinone while DCBQ mainly accepts electrons from Q_A by replacing plastoquinone at the Q_B site (see e.g., [35, 40]). DCBQ has also been used as an electron acceptor of inactive PSII centres of isolated spinach chloroplasts [12]. The electron transfer and exchange reactions at the Q_B site were also studied with chlorophyll *a* fluorescence relaxation after single-turnover flashes. Fluorescence relaxation in the submillisecond region is related to electron transfer from Q_A^- to Q_B or Q_B^-, and a slow millisecond phase to exchange reactions at the Q_B site (see e.g., [33]). The millisecond phase has been interpreted to reflect the exchange reaction in AR [25].

The oxygen-evolving capacity of both the autotrophic deletion mutant PD and heterotrophic strains CD and PCD was found to be approximately one fourth of that of AR control cells when monitored with DCBQ as the artificial electron acceptor. When using DMBQ as electron acceptor, even more drastic loss (90%) in the oxygen-evolving capacity of the heterotrophic mutants CD and PCD was observed. In contrast, the oxygen evolving capacity of the autotrophic strain PD was found to be independent of the artificial electron acceptor used. The point mutations E231D and E243Q had no significant effect on the oxygen-evolving capacity of the cells (Table 3). The LC strain was unable to evolve any oxygen in accordance with no assembled PSII centres observed in the Deriphat gels (data not shown).

Table 3. Oxygen-evolving activity of the *Synechocystis* 6803 site-specific mutants with two different quinone electron acceptors. For designation of the mutant strains see Fig. 1. Values are means from three independent experiments. Mean value (± standard error of the mean, SE).

strain	μmol O_2 (mg Chl)$^{-1}$ h^{-1}	
	DCBQ	DMBQ
AR	345 ± 10	317 ± 9
PD	98 ± 5	75 ± 4
CD	122 ± 4	22 ± 0
PCD	120 ± 8	16 ± 1
LC	0	0
E231D	356 ± 57	274 ± 16
E243Q	441 ± 32	423 ± 63

The relaxation of chlorophyll fluorescence after a single turnover pulse exhibited wild-type behaviour in the AR strain. The deletion mutants PD, PCD and CD showed a slow relaxation which was almost entirely in the millisecond and slower time domains. The relaxation was slightly faster in PD than in PCD and CD. No flash-induced variable fluorescence was detected in LC (Fig. 6).

Degradation rates of the mutated D1 proteins under growth conditions

Degradation rates of the D1 protein under growth conditions were determined by pulse-labelling the cells for 45 min with ^{35}S-methionine and thereafter monitoring the chase in radioactivity in the presence of unlabelled methionine. An example of such an autoradiogram for the cleavage region deletion mutant CD is presented in Fig. 7. Fitting the D1 protein degradation results to the first-order equation revealed a half-life of approximately three hours for the D1 protein in the PCD strain and the AR control strain, while the D1-protein half-life for the PD and CD deletion mutants and in the E231D and E243Q point mutants was clearly shorter than in the control strain (Table 4). Because the band detected by D1-protein-specific antibody was quite diffuse in the PCD strain (Fig. 3) making it thus somewhat

Table 4. Half-life of the D1 protein in the site-specific D-E loop mutants of *Synechocystis* 6803 under growth conditions. Autoradiograms from the pulse-chase experiments were analyzed with a laser densitometer, and the results were fitted to first-order kinetics to determine the half-life ($T_{1/2}$) of the D1 protein. Degradation of the D1 protein in the deletion mutants PD, CD and PCD and in the control strain AR was measured in the presence of glucose, whereas experiments with the point mutants E231D and E243Q were performed under autotrophic conditions. The results are expressed as the means ± SE from two to four independent experiments.

Strain	$T_{1/2}$, h
AR	3.3 ± 0.3
PD	2.0 ± 0.1
CD	1.8 ± 0.3
PCD	2.7 ± 0.3
E231D	1.6 ± 0.4
E243Q	2.2 ± 0.2

difficult to localize the D1 band in the autoradiograms, the D1-degradation rate was also verified by immunoblotting in the presence of lincomycin. Quantitative immunoblotting revealed similar D1-degradation rates for both the PCD strain and the AR control strain (data not shown), thereby confirming that the correct band had been studied in the autoradiographic procedure.

psbA *and* psbD *transcript levels of the mutants*

As shown in Fig. 8, the steady-state amounts of *psbA-2* and *psbD* transcripts under growth-light conditions were considerably higher in the PD, CD and LC strains than in the AR or PCD strains. The amount of *psbA-2* mRNA in E231D and E243Q did not differ from the *psbA-2* transcript level of AR (data not shown). We have earlier shown [50] that under growth conditions the high level of *psbA* transcripts in the D1 protein mutant strain CA1 [(ΔE242-244);E241H] resulted from high transcriptional activity of the *psbA-2* gene, not from increased stability of the mRNA. To study the effects of a wider range of light intensities, the half-life of the *psbA-2* mRNA was measured in the LC and control AR strains both under high (1500 μmol photons m^{-2} s^{-1}) and very low (3 μmol photons m^{-2} s^{-1}) fluence rates. Notably, the half-life of the *psbA-2* mRNA for both LC and AR was found to be similar, ca. 15 min, and independent of PPFD. This implies that also under growth conditions the *psbA-2* gene is transcribed more actively in LC than in the control strain.

Finally, to determine whether the variation in *psbA-2* transcript levels was related to photosynthetic electron transfer, BG-11 growth medium was supplemented with 10 μM DCMU. As demonstrated in Fig. 9, the steady-state amount of the *psbA-2* transcripts in wild-type *Synechocystis* 6803 was not affected by blocking PSII electron transfer with DCMU.

Discussion

Effects of the D-E loop mutations on the assembly and function of PSII

The D-E loop of the D1 protein is known to be longer than that of the L subunit of rhodobacteria [41, 45, 47, 54]. In this study, we have constructed deletion strains LC (ΔT227-Y246) and PCD (ΔR225-V249) to make the D-E loop of the D1 protein resemble the corresponding loop in the L subunit. Both of these deletion mutants were incapable of photoautotrophic growth, clearly indicating that this extension is an absolute requirement for the proper function of oxygenic PSII.

Although the deletions constructed in the heterotrophic strains LC and PCD are rather similar both in size and location, the electron transfer properties and assembly of PSII in these strains differ drastically. In the LC strain, neither PSII assembly nor oxygen-evolution activity were observed under any of the conditions studied (Figs. 4 and 6, Tables 2 and 3). Conversely, the PCD strain was found to contain high levels of the D1 protein (Fig. 3) and other PSII core polypeptides (Table 2). This is not only surprising since PCD has a larger deletion than that of LC, but also since the deletion in PCD encompasses within it a deletion identical to that constructed in the LC strain (Fig. 1). It can be concluded that the proper surrounding of amino acids $NIV_{247-249}$ is more crucial than the total absence of those amino acids for the stability of the D1 protein and therefore also for the assembly of PSII. Moreover, our results indicate that the PSII centres in the PCD strain exist as dimers (Fig. 5), which are thought to

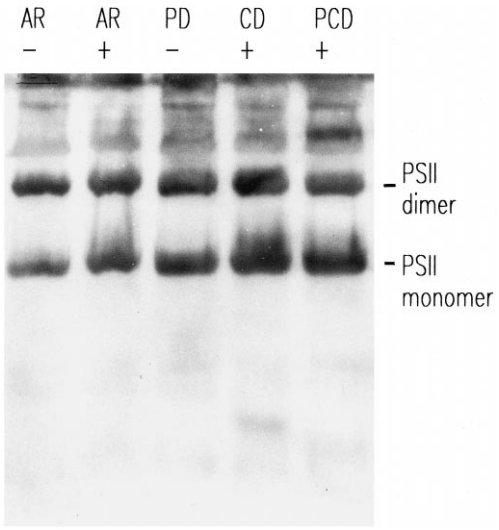

Figure 5. D1-protein immunoblot after non-denaturating Deriphat PAGE of dodecyl-β-D-maltoside solubilized thylakoid membranes of the *Synechocystis* 6803 deletion mutants. Mutant strains are indicated on the top of each lane (for specifications, see Fig. 1). (+) denotes growth of the cells in the presence and (–) in the absence of 5 mm glucose.

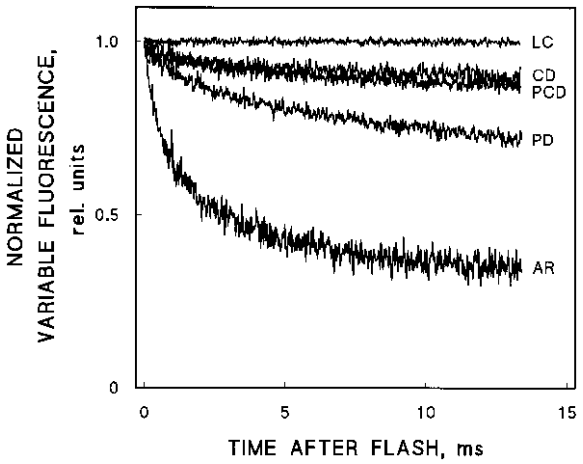

Figure 6. Chlorophyll *a* fluorescence relaxation curves of the mutant strains and AR control strain, measured with a PAM fluorometer at 20 °C. The curves have been normalized to the flash-induced variable fluorescence in each experiment, except for LC, which has no variable fluorescence.

be the functional form of PSII in photosynthetic membranes [2, 3].

Like PCD, also the other cleavage region mutant CD (ΔG240-V249) had lost the capability for autotrophic growth and resembled PCD in many other functional and structural properties of PSII (Figs. 3, 5 and 6, Tables 2 and 3). The results from the oxygen evolution

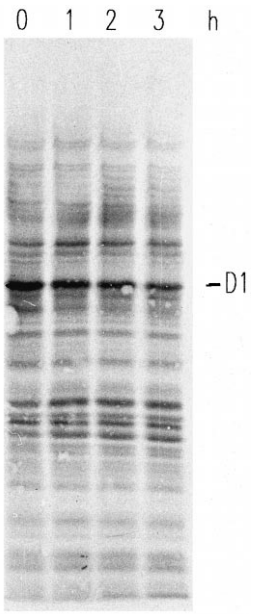

Figure 7. Autoradiogram of thylakoid membrane polypeptides in *Synechocystis* 6803 cleavage region mutant CD after a 45 min pulse *in vivo* (0 h) in the presence of ^{35}S-methionine and a chase for 1, 2 and 3 h in the presence of non-radioactive methionine, both under growth conditions.

experiments (Table 3) indicate that the CD and PCD mutants may have a major block in the plastoquinone exchange reaction at the Q_B site, as the steady-state electron transfer rates to DMBQ were much lower than the rates of electron transfer to DCBQ. The chlorophyll *a* fluorescence relaxation curves (Fig. 6) of the CD and PCD strains are composed of slow phases only, confirming that also electron transfer from Q_A^- to Q_B is slow in the CD and PCD strains. Interestingly, the heterotrophic mutant $\Delta YNIV_{246-9}::E_{244-5}$ constructed by Kless and coworkers [17] contained a short deletion in the cleavage region but did not evolve any oxygen when DMBQ was employed as the electron acceptor. PSII assembly in this mutant, however, was not tested. Nevertheless, another deletion mutant CA1 [Δ(E242-E244); Q241H] is able to grow photoautotrophically [25] and contains levels of functional PSII centres similar to those in wild-type cells [50]. These results indicate that the native C-terminal portion of the cleavage region (see Fig. 1) is required for proper assembly and function of PSII. Furthermore, N247, which is deleted in both CD and PCD but not in CA1, has earlier been implicated in Q_B binding (see [17, 48]) and indeed, our results are in line with this suggestion.

The PD strain (ΔR225-F239), with 15 amino acids deleted from the region of the PEST-like sequence, contains high amounts of all five PSII polypeptides examined in this study, evolves oxygen and grows photoautotrophically. These findings are consistent with the properties of the PEST-deletion mutant ΔPEST (Δ226-233) of *Synechocystis* sp. PCC 6803 described recently [28]. It has also been shown that *Synechocystis* sp. PCC 6803 mutants with shorter deletions in the PEST-like region of the D1 protein are fully capable of photoautotrophic growth [17].

The chlorophyll *a* fluorescence relaxation (Fig. 6) and DCBQ-dependent oxygen evolution (Table 2) show that electron transfer from Q_A^- to Q_B is severely slowed down in the PD strain. In contrast, the rate of electron transfer between Q_A^- and Q_B is not drastically altered in the mutant ΔPEST [28], indicating that the larger (15 amino acids) deletion in the PD strain is more harmful for Q_A^- to Q_B electron transfer than the eight amino acid deletion in ΔPEST. On the other hand, steady-state electron transfer to DMBQ was near to the rate of electron transfer to DCBQ both in PD and ΔPEST, suggesting that the plastoquinone exchange reaction at the Q_B site is less severely affected in both PEST region mutants than in CD and PCD, in which the cleavage region has been deleted (Table 3). Additionally, the slightly faster millisecond-scale fluorescence relaxation in PD compared to CD and PCD confirms that the reason why PD but neither CD nor PCD can grow autotrophically, probably is a faster plastoquinone exchange reaction at the Q_B site in PD. The autotrophic growth rate of the PD cells was only slightly slower than that of the control strain AR (data not shown), indicating that the rate of oxygen evolution measured with artificial electron acceptors has no direct correlation with growth rates, as has also been found in studies with other *Synechocystis* 6803 mutants [17, 52].

Degradation of the D1 protein in the D-E loop mutants

Perturbations at the acceptor side of PSII were not related in any logical way to the degradation rate of the D1 protein under growth conditions. Therefore, no direct relationship apparently exists between PSII function, e.g. the occupancy of the Q_B site [38, 46], and the rate of D1 protein degradation (Tables 3 and 4) in the D-E loop deletion mutants. The degradation rate of the D1 protein was faster in the PD and CD deletion mutants and in the E231D and E243Q point mutants under growth conditions than in the control strain AR, although the electron transfer rates of the two latter mutants did not differ from those of the control strain AR. Similarily, the ΔPEST deletion mutant has been reported to exhibit faster turnover rate of the D1 protein as compared to the control strain TC31 [28]. However, ΔPEST mutation was introduced into the *psbA-3* gene [28], which has been shown to produce less than 10% of the total *psbA* mRNA in wild-type *Synechocystis* 6803 cells [5, 22] thus making the direct comparison of *psbA* gene expression in the PD and ΔPEST strains difficult.

It has earlier been suggested that degradation of the D1 protein may begin after a protease has recognized the PEST-like sequence in the D-E loop of the D1 protein [13]. Our results are consistent with the other results [17, 28, 49], which clearly indicate that the PEST-like sequence is not absolutely required for D1 degradation. The PEST-like sequence might, however, be required for optimal function of the D1-specific protease under stress conditions. Furthermore, deletion of the amino acid stretch 240–249 or an even longer stretch 225–249 including the putative primary cleavage region of the D1 protein [13, 39, 44] did not render the D1 protein resistant to proteolytic degradation. It can thus be concluded that this specific region of the D-E loop in the D1 protein, as such, is not a requirement for D1 degradation.

The fast degradation rate of the D1 protein in several D-E loop mutants (Table 4, [18, 28–30, 49]) may be due to aberrant folding of the mutated proteins. This notion is supported by this study and the work of [49] showing that even modifications as small as point mutations in the D-E loop may result in conformational changes in the D1 protein, seen as mobility shifts in SDS-PAGE. Such conformational changes could lead to proteolytic cleavage of the D1 protein by unspecific household protease(s). The degradation rate of the D1 protein in the PCD strain, however, did not differ from that of the control, although major conformational changes were evident in the D-E loop. Nevertheless, we speculate that the 'cleaning' protease does not, for some yet unknown reason, recognize the D1 protein of the PCD strain to be impaired. Cleavage of newly synthesized but aberrantly folded D1 proteins has been postulated to occur in the D-E loop [16], whereas the specific site of light-induced D1 protein cleavage under *in vivo* conditions is still under discussion [6, 13, 15].

Although D1 is stably incorporated into the membranes of the PCD strain, it is unclear why the mutated D1 protein cannot be detected in the LC strain. We

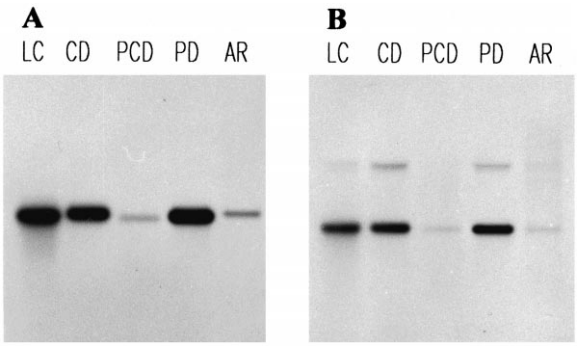

Figure 8. Steady-state transcript levels of the *Synechocystis* 6803 deletion mutants under growth conditions. A. *psbA-2* transcripts: *psbA-2* mRNA of LC and PCD strains containing the largest deletions show faster electrophoretic mobility than those of other strains. B. *psbD* transcripts. The upper band represents the transcript of the overlapping *psbD-1/psbC* genes and the lower band is a product of *psbD-2* gene. 5 μg of total RNA was loaded in each well. The membrane was first hybridized with *psbA-2* probe, then washed and reprobed with *psbD*.

still do not know whether the D1 protein is synthesized in the LC mutant and then rapidly degraded after insertion, or whether the synthesis of the D1 protein is blocked. There is, however, no obvious reason to expect inhibition of *psbA-2* mRNA translation in LC, since no differences could be found between the LC and AR strains when the untranslated upstream region of the *psbA-2* gene was sequenced. We therefore speculate that the amino acid sequence $NIV_{247-249}$, which is present in the mutant LC but not in PCD, might act as a determinant for rapid proteolysis if the native conformation of the loop has drastically changed.

Apparently, the loss of ability to synthesize stable D1 protein in the LC strain resulted in rapid degradation of other PSII polypeptides as well (Table 2). However, in accordance with results on the D1-less *Synechocystis* 6803 mutant [27], the thylakoids of the strain LC were found to contain high amounts of free cyt b_{559} and 43 kDa chlorophyll *a* binding protein. Several mutants of a single PSII gene have previously been reported to result in the depletion or decrease of several other PSII components from the thylakoid membranes [27, 51, 55]. The inability to synthesize one of the PSII proteins [55] does not lead to a suppression of transcription and translation of other PSII proteins but may disrupt the stability of these proteins.

Elevated psbA-2 *transcript level in the D-E loop mutants*

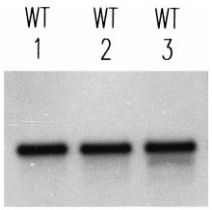

Figure 9. Northern blot analysis of wild-type (WT) *Synechocystis* 6803 cells using *psbA-2* DNA as a probe. Cells were grown in BG-11 medium (lane 1), in BG-11 medium supplemented with glucose (lane 2) and in BG-11 medium supplemented with both glucose and DCMU (lane 3) for two days before isolation of RNA.

Although the amount of *psbA-2* transcripts generally increases with increasing irradiance [23], the present results (Fig. 8) and earlier work with other D-E loop mutant strains in our laboratory [50] clearly indicate that neither the light intensity as such nor the activity of the electron transfer chain (Fig. 9) provide direct signals for activation of the *psbA-2* gene. Under growth light conditions, the amounts of *psbA-2* and also *psbD* transcripts in the PD, CD and LC mutant strains were several-fold higher than in the AR control strain (Fig. 8). This increase clearly results from transcriptonal activation, not from differences in *psbA-2* mRNA stability, as we have also shown earlier for another D-E loop mutant CA1 [47]. These results are in agreement with an earlier study [24], which demonstrated that inhibition of linear electron transfer at the acceptor side of PSII and cyclic electron transfer around PSI did not prevent the light-stimulated accumulation of *psbA* transcripts. Considering also the other properties of the D-E loop mutants presented in this study (see also [50]), we assume that the transcriptional and translational activities of PSII genes coincide with the apparent need for PSII components. Activation of *psbA* gene expression only is typically observed when the D1 protein of PSII undergoes rapid turnover. However, in the mutants with large deletions, transcription of several PSII genes is probably enhanced to increase the number of PSII centres in order to compensate for impaired PSII function, resulting in an elevated PSII/PSI ratio. The signals for these two types of transcriptional activation remain to be elucidated.

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