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Deletions in the luminal domains of the D1 protein that prevent the assembly of photosystem II in *Synechocystis* sp. PCC 6803

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Introduction

The D1 photosystem II (PSII) reaction center protein contributes hydrophilic domains to the water-oxidizing complex (Barber *et al.*, 1999; Zouni *et al.*, 2001). In particular, the recent crystal structure of PSII from *Synechococcus elongatus* has confirmed that luminal domains of D1 are in close contact with the manganese cluster of the enzyme (Zouni *et al.*, 2001). Extensive mutagenesis studies targeting these domains have been performed to discover which residues are essential for the ligation of manganese and calcium at the active site, and several carboxylate and histidine residues have been identified (reviewed in Debus (2001) and Diner (2001)). However, the detailed structure of the protein environment surrounding the manganese cluster has not been established. In addition, the introduction of segment deletions into luminal domains of D1 has indicated that the accumulation of the D2 and CP47 proteins of PSII requires the insertion, but not necessarily the accumulation, of D1 (Salih *et al.*, 1996). In this study we have created short amino acid deletions to identify important regions in the luminal domains of the D1 protein in *Synechocystis* sp. PCC 6803. These deletions were targeted at amino acids with alkoxo and phenoxo groups which may also participate in cofactor binding (Debus, 1992).

Materials and methods

Mutations were introduced into the *psbA2* gene using the Muta-Gene Phagemid *in vitro* mutagenesis kit (Bio-Rad) and mutants were confirmed by PCR analysis and sequencing. Photoautotrophic growth rates in liquid BG-11 were determined by measuring the optical density of cultures at 730 nm. Oxygen evolution was measured in BG-11 containing 25 mM HEPES-NaOH, pH 7.5 at 30°C and at 10 µg/ml chlorophyll *a*. Actinic light was provided through a Melles Griot OG 515 yellow glass filter at 700 and 7000 µE.m⁻².s⁻¹. Fluorescence measurements were performed on an OCCAM Technologies kinetic fluorimeter using the protocol of Nixon and Diner (1992). Immunoblotting was performed on isolated thylakoids. Additional details are given in Clarke and Eaton-Rye (2000).

Results

We have constructed a *psbA* mutation system consisting of a *psbA* triple-deletion strain (*psbA*-Del) and a complementary mutagenesis system that allows us to introduce mutations into the *psbA2* gene (W. Nicoll and J. Eaton-Rye, unpublished). As a result we have introduced seven deletions into the luminal regions of D1 targeting at least one conserved Ser or Tyr in each deletion. The following strains were created: control; Δ(P66-S70); Δ(Y73-S79); Δ(V82-S86);

$\Delta(A99-S101)$; $\Delta(Y107-G109)$; $\Delta(L174-S177)$ and $\Delta(F302-S305)$. The location of each of the deletions in each is shown in Fig. 1.

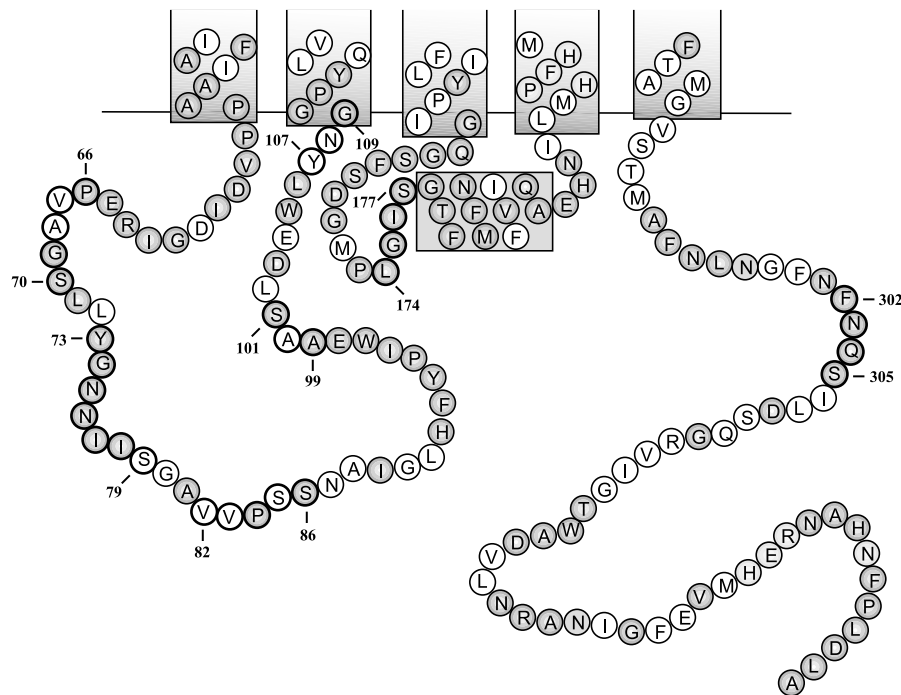


Fig. 1. The D1 luminal domains in *Synechocystis* sp. PCC 6803. Residues conserved in all known D1 sequences are shaded. Deletions introduced in this study are indicated in bold together with the numbers of the first and last residue in each deleted segment. The A-B domain (Pro-57 to Asn-108), the C-D domain (Gln-165 to Ile-192) and the C-terminal extension (Val-290 to Ala-344) are shown.

The results shown in Fig. 2A demonstrate that photoautotrophic growth was restored to the *psbA*-Del strain by transformation with a plasmid containing a wild-type *psbA2* with a kanamycin-resistance cassette inserted downstream. In contrast, none of the deletion strains were able to grow photoautotrophically. Photosystem II activity in all strains was assessed through measurements of oxygen evolution from whole cells under continuous actinic light (Fig. 2B). At $700 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ the control strain exhibited an initial rate of $326 \mu\text{mol O}_2 \cdot (\text{mg of chlorophyll})^{-1} \cdot \text{h}^{-1}$ compared to $222 \mu\text{mol O}_2 \cdot (\text{mg of chlorophyll})^{-1} \cdot \text{h}^{-1}$ for the wild type. A similar result was obtained at $7000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (data not shown). This suggests that the chlorophyll/PSII ratio may be different in the two strains. However, none of the mutants evolved oxygen suggesting that no photochemically active PSII centers were present.

Figure 2C illustrates that the variable chlorophyll *a* fluorescence yield of the control was similar to that of the wild type while all luminal deletion mutants exhibited little or no variable fluorescence yield, also suggesting that PSII assembly in these mutants was impaired. To further assess PSII assembly, immunoblots of thylakoid membranes were probed with antibodies raised against D1 and D2 (Fig. 2D). The strains carrying deletions in the luminal loops exhibited an almost complete lack of the D1 protein as well as reduced amounts of D2 when compared to the control. It is possible that the luminal deletions in this study are interfering with translational regulation that result in the rapid break down of the mutant D1 protein (Tyystjärvi *et al.*, 2001). In contrast, the C-terminal domain mutant, $\Delta(F302-S305)$ was found to have significant levels of D1 and D2 in the membrane that were approximately

60% and 80% of the levels in the control strain, respectively. However, the $\Delta(F302-S305)$ mutant appears to retain its full length D1 protein (the origin of the D1 doublet seen in Fig. 2D is still under investigation). Moreover, the presence of high levels of D2 in the $\Delta(F302-S305)$ strain suggests that PSII centers are being assembled despite the quenched fluorescence yield. However, no PSII centers were detected by diuron replaceable [^{14}C]atrazine binding (data not shown).

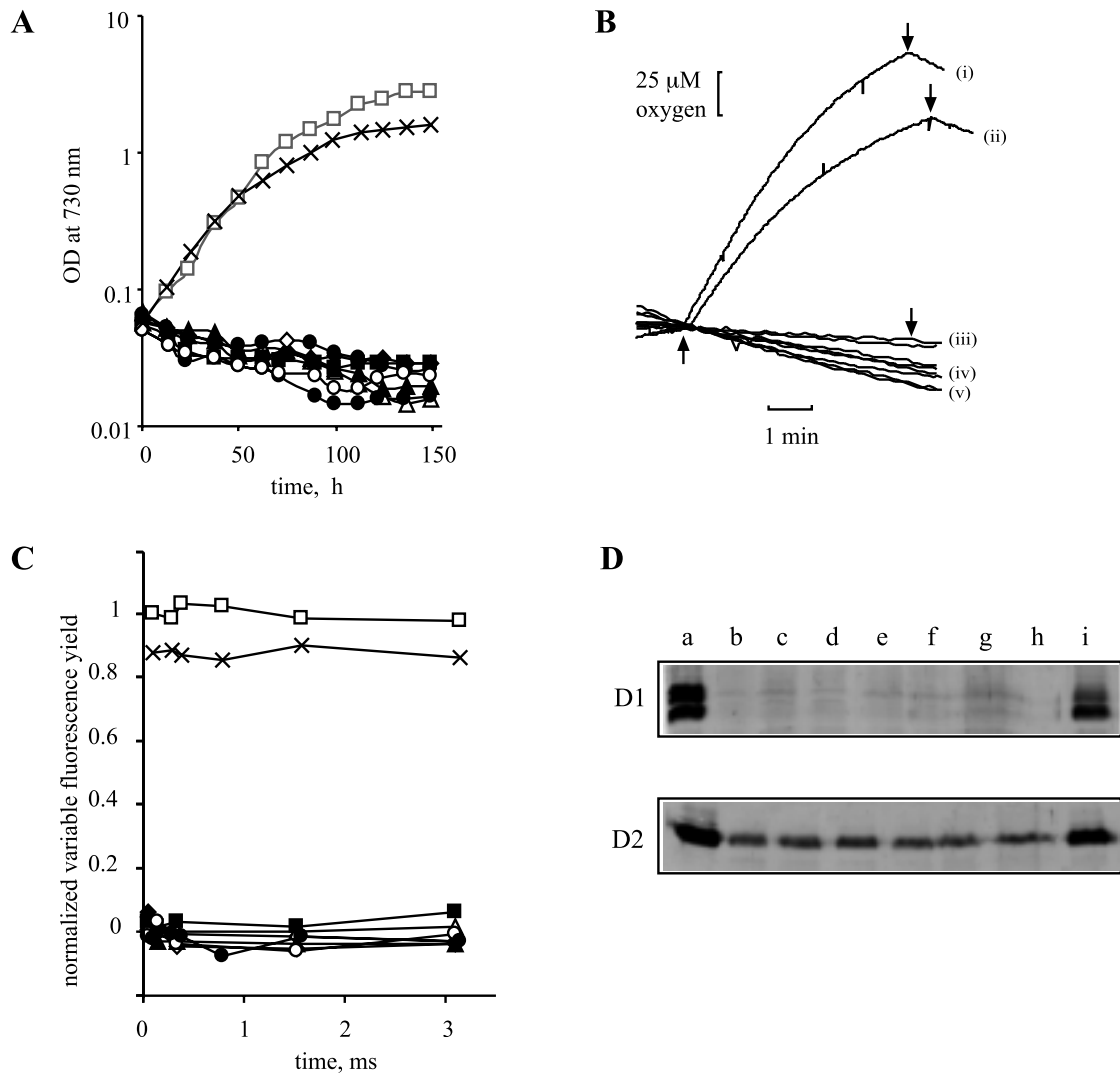


Fig. 2. A. Photoautotrophic growth curves of *Synechocystis* sp. PCC 6803 strains as measured by the optical density at 730 nm in BG-11. Control (open squares); wild type (crosses); $\Delta(P66-S70)$ (closed squares); $\Delta(Y73-S79)$ (open triangles); $\Delta(V82-S86)$ (open diamonds); $\Delta(A99-S101)$ (closed circles); $\Delta(Y107-G109)$ (closed triangles); $\Delta(L174-S177)$ (open circles) and $\Delta(F302-S305)$ (closed diamonds). B. Traces of oxygen concentration as determined with a Clark electrode measured at a light intensity of $700 \mu\text{E m}^{-2} \text{s}^{-1}$. Arrows indicate light on and off. Control (i); wild type (ii); *psbA*-Del and $\Delta(V82-S86)$ (iii); $\Delta(P66-S70)$, $\Delta(Y73-S79)$, $\Delta(Y107-G109)$ and $\Delta(F302-S305)$ (iv); $\Delta(A99-S101)$ and $\Delta(L174-S177)$ (v). C. Variable chlorophyll *a* fluorescence yield normalized to a control value of 0.68. Control (open squares); wild type (crosses); $\Delta(P66-S70)$ (closed squares); $\Delta(Y73-S79)$ (open triangles); $\Delta(V82-S86)$ (open diamonds); $\Delta(A99-S101)$ (closed circles); $\Delta(Y107-G109)$ (closed triangles); $\Delta(L174-S177)$ (open circles) and $\Delta(F302-S305)$ (closed diamonds). D. Immunoblots of thylakoid membranes probed with either an antibody raised against D1 or D2. Control (a); *psbA*-Del (b); $\Delta(P66-S70)$ (c); $\Delta(Y73-S79)$ (d); $\Delta(V82-S86)$ (e); $\Delta(A99-S101)$ (f); $\Delta(Y107-G109)$ (g); $\Delta(L174-S177)$ (h) and $\Delta(F302-S305)$ (i).

Discussion

A previous study has introduced two segment deletions into the A-B luminal loop of D1. These mutants were $\Delta(V58-D61)$ and $\Delta(D103-G109);G110R$, located adjacent to helices A and B, respectively (Salih *et al.*, 1996). The integration of the D1 protein into the thylakoid membrane in these strains was inhibited and this was accompanied by the virtual absence of either D2 or CP47. However, the $\Delta(N325-E333)$ mutant did integrate into the membrane, albeit with reduced stability, and near wild-type levels of D2 and CP47 accumulated. This result is interesting since His-332 and Glu-333 influence the assembly or stability of the manganese cluster (reviewed in Debus (2001) and Diner (2001)). In contrast, the D2 protein was detected in all of our mutants even though only trace levels of D1 were present in our luminal loop deletion strains. Interestingly, the C-terminal domain mutant, $\Delta(F302-S305)$, was found to have significant levels of D1 and D2 in the membrane. Based on the results of Salih *et al.* (1996) we would expect both the CP47 and CP43 chlorophyll *a*-binding proteins to be present in this mutant and therefore diuron replaceable [^{14}C]atrazine binding was performed as an additional assay for assembled PSII centers; however, none were detected. This deletion is in a conserved area of the long C-terminal extension and may be involved in protein-cofactor or protein-protein interactions that are essential for the assembly of the PSII complex.

In conclusion, each of the deletions introduced in this study incorporated the removal of amino acids with side chains possessing alkoxo or phenoxo groups; and, since each deletion prevented the assembly of PSII, additional point mutations might be informative. To date only Pheo substitutions at Tyr-73 and Tyr-107 have been tested, and both were found to be similar to wild type (Chu *et al.*, 1995). We have created five segment deletion mutants in the A-B luminal loop of D1 and one deletion mutant in the C-D loop. The D1 protein was virtually absent from these mutants although the D2 protein was present. An additional mutant, $\Delta(F302-S305)$, was found to have appreciable levels of both D1 and D2 but no assembled PSII centers were detected in this strain. It is likely that the phenotype of these mutants arises from structural perturbations; however, a specific role for individual residues within these regions cannot be excluded.

Acknowledgments

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