S13-004

Mutations between Gly-429 and Thr-436 in loop E of the chlorophyll *a*-binding protein CP47 characterized in the presence or absence of the membrane-extrinsic proteins of the water-oxidizing complex of photosystem II

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Keywords: CP47, oxygen evolution, photosystem II, mutants, *Synechocystis*

Introduction

The protein environment of the water-oxidizing complex (WOC) of photosystem II (PSII) has yet to be fully defined although the reaction center D1 protein has been shown to contribute to the ligation of manganese at the active site, and the PSII-O protein, encoded by the *psbO* gene, is required to stabilize the complex *in vivo* (Debus, 2000). In the chlorophyll *a*/*b*-containing eukaryotes the PSII-P and PSII-Q proteins, encoded by *psbP* and *psbQ*, respectively, are also present on the lumenal face of the complex and participate in the binding of calcium and chloride cofactors (Seidler, 1996). However, in all non-green plants, and in cyanobacteria, the *psbP* and *psbQ* genes are not found and two different proteins, PSII-U, encoded by *psbU*, and PSII-V (a *c*-type cytochrome) encoded by *psbV*, are present. In addition, it has been shown in the red algae that a 20 kDa protein also participates in the binding of PSII-U and PSII-V to the complex (Enami *et al*., 1998). Therefore it is possible to distinguish between PSII-P type and PSII-V type photosystems based on the composition of the membrane-extrinsic proteins (e.g., Morgan *et al*., 1998).

In a 3D map of PSII from spinach the chlorophyll *a*-binding core antenna proteins, CP47 and CP43, are found adjacent to the D2 and D1 proteins, respectively, and the PSII-O protein is found towards the CP47/D2 side, although it is also associated with hydrophilic domains arising from the C-terminal membrane-spanning helices of D1. In contrast, the PSII-P and PSII-Q proteins are positioned over the N-terminal domain of D1 (Nield *et al*., 2000). Furthermore, the PSII structure from *Synechococcus elongatus* has also located the position of PSII-O and identified the PSII-V protein on the CP43 side of the CP47/D2/D1/CP43 complex (Zouni *et al*., 2001). However, the position of PSII-U could not be assigned due to an inability to distinguish between loop regions arising from the membrane-spanning domains, as well as unassigned secondary structure associated with PSII-O and PSII-V.

Whereas, the minimum number of proteins required for stable water-splitting by PSII includes CP47, CP43, D1, D2 and cytochrome b_{559}, the light-driven assembly of an active manganese cluster has been reported in a subcomplex lacking CP43, which suggests an important role for CP47 (Buchel *et al*., 1999). The CP47 protein has six membrane-spanning domains, and the most significant hydrophilic domain in the lumen is loop E connecting helices V and VI (Bricker (1990) and see Fig. 1). We have previously studied the effects of removing PSII-O and PSII-V from photoautotrophic mutants carrying short segment deletions in CP47 and identified regions in loop E that responded differently to the removal of these proteins (Morgan *et al*., 1998). Here we have extended this comparison to include the removal of PSII-U. This has led to the discovery that the absence of PSII-U generates a

phenotype that is similar to that observed when *psbO* is deleted in these strains and this has also uncovered a chloride effect on PSII activity when CP47:Phe-430 is deleted.

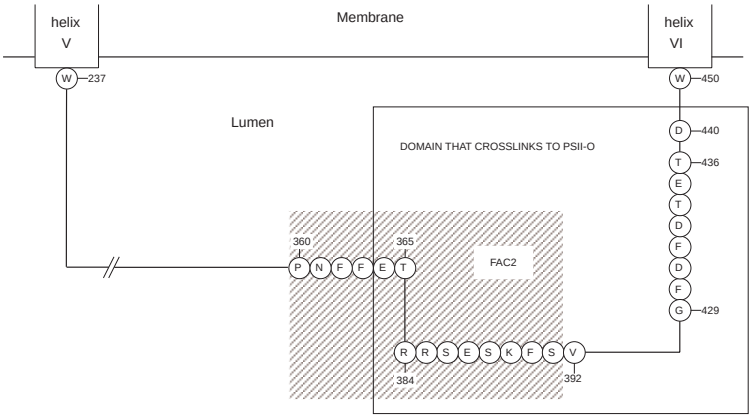

Fig. 1. The large lumen-exposed hydrophilic loop located between helix V and helix VI of CP47. The domain between Glu-364 and Asp-440, that has been shown to cross-link to the N-terminal 76 amino acids of the PSII-O protein, is shown by an open box (Odom and Bricker, 1992). The domain from Pro-360 to Ser-391, indicated by the hatched box, is an epitope for the monoclonal antibody FAC2 (Bricker and Frankel, 1987; Frankel and Bricker, 1989). The two regions, Arg-384 to Val-392, and Gly-429 to Thr-436, that have been used to probe interactions with PSII-O, PSII-U and PSII-V, are indicated. The sequences are for *Synechocystis* sp. PCC 6803.

Materials and methods

The oligonucleotide-directed mutations in the *psbB* gene encoding CP47, the interruption of *psbO*, and the deletion of *psbV*, were performed as described in Morgan *et al*. (1998). The interruption of the *psbU* gene resulting in the removal of the PSII-U protein was carried out as described in Clarke and Eaton-Rye (1999). The methods for the measurements of chlorophyll fluorescence yield, herbicide binding, oxygen evolution, and photoautotrophic growth, as well as the transformation and verification of cyanobacterial strains, are also described in Morgan *et al*. (1998). Western blotting was carried out as described in Clark and Eaton-Rye (2000). The control strain used in these experiments contained a kanamycin-resistance cassette located downstream of the *psbB* gene and was identical to the wild type except for the antibiotic-resistant phenotype (Eaton-Rye and Vermaas, 1991).

Results

The position of the segment deletions in loop E of CP47 that were utilized in this study have been shown in Fig. 1. The removal of PSII-O in the Δ(G429-T436) mutant prevented the assembly of PSII, whereas the Δ(R384-V392):ΔPSII-O mutant remained photoautotrophic; however, both the Δ(R384-V392):ΔPSII-V and the Δ(G429-T436):ΔPSII-V mutants were obligate photoheterotrophic strains (Morgan *et al*., 1998). When the PSII-U protein was removed to create the Δ(R384-V392):ΔPSII-U mutant the phenotype remained similar to that of the Δ(R384-V392) strain (data not shown). Therefore, as expected, Fig. 2A shows that the control and control:ΔPSII-U strains both had photoautotrophic doubling times of ~12 h. In contrast, the doubling time of the Δ(G429-T436) mutant was extended to ~18 h and the removal of PSII-U in this strain resulted in the disruption of stable PSII assembly and the mutant remained in a stationary phase. However, trace levels of D1 and D2 could still be detected and the centers that were assembled were able to evolve oxygen at a rate that was ~30% of the control, although these centers were sensitive to photoinactivation (Table 1).

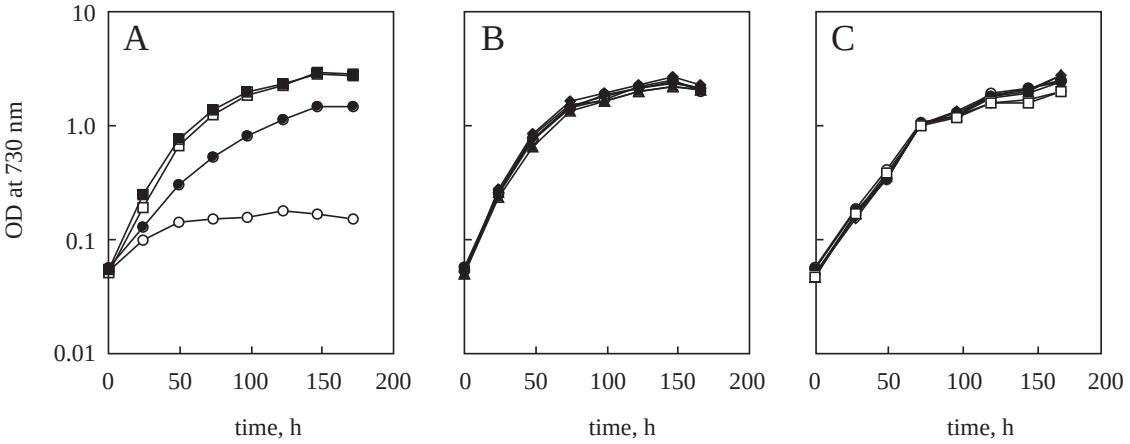

Fig. 2. Photoautotrophic growth curves of *Synechocystis* sp. PCC 6803 strains as measured by the optical density at 730 nm in BG-11. A. Control (solid squares), control:ΔPSII-U (open squares), Δ(G429-T436) (solid circles) and Δ(G429-T436):ΔPSII-U (open circles). B. D431A (solid circles), D431E (solid diamonds), D431N (solid triangles), D433A (open circles). D433E (open diamonds) and D433N (open triangles). C. Control:ΔPSII-V (open squares), D431A:ΔPSII-V (solid circles), D431E:ΔPSII-V (solid diamonds), D431N:ΔPSII-V (solid triangles), D433A:ΔPSII-V (open circles). D433E:ΔPSII-V (open diamonds) and D433N:ΔPSII-V (open triangles).

The comparison of 49 different sequences between Gly-429 and Thr-436 of CP47 in Fig. 3 revealed that charged residues were conserved at positions 431 and 433 in this segment. Mutations were created at these positions changing Asp to Ala, Glu or Asn at both positions. As shown in Fig. 2B each of these strains had a photoautotrophic doubling time similar to the control. Since we have observed other charged residues in Loop E of CP47 that, when mutated, only created a lethal phenotype when the PSII-V protein was removed, we deleted PSII-V in each of the strains (e.g., Morgan *et al*., 1998). However, each mutant grew in a similar fashion to the control:ΔPSII-V strain in Fig. 2C. In addition, we introduced mutations at positions Pheo-430 and Thr-436. Again, mutations at these positions, in the presence or absence of the extrinsic proteins, exhibited phenotypes that were similar to the respective control strains. These data are summarized in Table 2. We also checked to see if any of the mutants were sensitive to the removal of chloride as the Δ(G429-T436) strain was unable to

Table 1. Summary of the characterization of the control, control:ΔPSII-U, Δ(G429-T436) and Δ(G429-T436):ΔPSII-U strains.

Strain	Herbicide binding[a] (Chl/PSII)	Abundance of PSII proteins[b]		Rate of oxygen evolution	TFI[c]
		D1	D2		
Control	560	++++	++++	280	st[d]
Control:ΔPSII-U	560	++++	++++	188	202
Δ(G429-T436)	1790	+++	++	168	198
Δ(G429-T436):ΔPSII-U	nd[e]	+	+	87	128

[a]Chl/PSII was determined by [^{14}C]atrazine binding in cell suspensions as described by Morgan *et al.* (1998). [b]Determined by immunoblots (++++) = wild-type level; (+++) = noticeably reduced; (++) = low level and (+) = trace. [c]Time to full photoinactivation (TFI) is the time (s) for oxygen evolution to be come completely inhibited during continuous actinic illumination. [d]Stable rate of oxygen evolution. [e]None detected.

grow photoautotrophically in a chloride-limiting medium (Morgan *et al*., 1998). However, no chloride effect was observed in these mutants (data not shown). Finally, we created the ΔF430, ΔD431, ΔD433 and ΔT436 strains and repeated the measurements in chloride-limiting media. The data in Fig. 4A,B showed that a bromide-reversible chloride effect was associated with the ΔF430 strain, but not the control or the ΔT436 mutant, and that this was not observed when the cells were grown photoheterotrophically in the presence of diuron: thus indicating that the ΔF430 strain was exhibiting a PSII-specific chloride effect. In Fig 4C the ΔF430 mutant was also found to lose its capacity for photoautotrophic growth when the PSII-V protein had been removed and, similarly, the ΔF430:ΔPSII-O was also impaired. In Fig. 4 the phenotypes of the ΔT436 mutants were similar to the corresponding control strains: as were the phenotypes of the ΔD431 and ΔD433 strains in the presence or absence of the membrane-extrinsic proteins (data not shown).

PSII-P type		429 436	**PSII-V type**		429 436
Dicotyledons (22)	*An*	IFELDRAT	*Cyanidium caldarium*	*Rh*	VFEFDRA**I**
Monocotyledons (5)	*An*	IFELDRAT	*Porphyra purpurea*	*Rh*	VFEFDRTT
Pinus thunbergiana	*Co*	IFELDRAT	*Amphidinium operculatum*	*Di*	IFSFDRKT
Ginkgo biloba	*Gi*	IFELDRAT	*Heterocapsa triquetra*	*Di*	IFTFDKKT
Zamia furfuracea	*Cy*	IFELDRAT	*Odontella sinensis*	*Ba*	VFEFDRT**S**
Marchantia polymorpha	*Br*	IFEFDRAT	*Guillardia theta*	*Cry*	V**L**EFDRTT
Chlamydomonas reinhardtii	*Ch*	IFEFDR**S**T	*Cyanophora paradoxa*	*Gl*	AFEFDRET
Chlorella vulgaris	*Ch*	IFEFDRAT	*Prochlorothrix hollandica*	*Cya*	AFDFDTET
Mesostigma viride	*Ch*	IFEFDRA**R**	*Anabaena (Nostoc)* sp. PCC 7120	*Cya*	IFEFDRET
Nephroselmis olivacea	*Ch*	**V**FEFDRAT	*Mastigocladus laminosus*	*Cya*	IFEFDRET
Euglena gracilis	*Eu*	IF**D**F**N**R**SI**	*Synechococcus* sp. PCC 7942	*Cya*	AFEFDTET
			Synechocystis sp. PCC 6803	*Cya*	GFDFDTET
			Thermosynechococcus elongatus	*Cya*	IFEFDTET
		* * *			* * *
Consensus (>90%)		IFE-DRAT	Consensus (>90%)		-F-FD--T

Fig. 3. Alignment of the amino acid sequences in loop E of CP47 between Gly-429 and Thr-436. The PSII-P type photosystems are from green algae and metaphytes where the water-oxidizing complex includes the PSII-P and PSII-Q proteins encoded by *psbP* and *psbQ*, respectively; and, the PSII-V type photosystems are from the red algae, chlorophyll *c*-containing eukaryotes and the cyanobacteria where PSII-V and PSII-U, encoded by *psbV* and *psbU*, are present but PSII-P and PSII-Q are not. The amino acid sequences were obtained form the Protein Data Base accessed via the National Center for Biotechnology Information. An asterisk indicates a residue that has been mutated in this study. The Asp-431 residue in *Synechocystis* sp. PCC 6803 was also mutated. The abbreviations are: *An*, Angiosperms; *Ba*, Bacillariophyta; *Br*, Bryophyta; *Ch*, Chlorophyta; *Co*, Conifer; *Cry*, Cryptophyta; *Cy*, Cycadophyta; *Cya*, Cyanophyta; *Di*, Dinophyta; *Eu*, Euglenophyta; *Gi*, Ginkgophyta; *Gl*, Glaucophyta, and *Rh*, Rhodophyta.

Discussion

The function of the PSII-U protein is not yet fully understood and the position of this protein in the 3.8 Å cyanobacterial PSII crystal structure has not been clarified (Zouni *et al*., 2000). When the PSII-U protein was removed in the Δ(R384-V392) mutant, which carries a segment deletion in the FAC2 epitope that is exposed following the removal of the membrane-extrinsic proteins in spinach, no change in phenotype was observed. However, removal of PSII-U in the background of the Δ(G429-T436) mutant resulted in a reduction in the level of assembled PSII centers to below the level that could be detected by herbicide binding. This suggests that the PSII-U protein was able to stabilize the perturbation that was introduced by the deletion in the Δ(G429-T436) mutant. To investigate the importance of the amino acids within this deletion we introduced substitutions or deletions at the highly conserved residues shown by an asterisk in Fig. 3 as well as at Asp-331. Putnam-Evans *et al*. (1996) had

Table 2. Characterization of *Synechocystis* sp. PCC 6803 strains containing mutations at Phe-430 and Thr-436 in the presence and absence of PSII-O, PSII-U and PSII-V.

Strain	Photoautotrophic Growth[a]	Rate of Oxygen Evolution[b]	TFI[c]	PSII Abundance Determined by Herbicide Binding[d]
Control	12	1.0	st[e]	1.0
ΔPSII-O	20	0.6	180	0.7
ΔPSII-U	12	0.8	190	1.0
ΔPSII-V	17	0.5	120	0.6
F430L	13	1.0	st	1.0
ΔPSII-O	18	0.7	160	0.5
ΔPSII-U	14	0.8	160	0.9
F430Y	13	1.0	st	1.0
ΔPSII-O	18	0.5	160	0.7
ΔPSII-U	14	0.9	st[f]	0.8
ΔPSII-V	18	0.7	220	0.6
T436A	14	1.0	st	0.9
ΔPSII-O	nd	0.6	180	0.8[f]
ΔPSII-U	nd	0.8	st[g]	0.8[f]
ΔPSII-V	nd	0.3	120	nd

[a]Doubling time in h. [b]Normalized to the control rate of 260 μmoles O_2 (mg of chlorophyll)$^{-1}$ h^{-1}. [c]Time to full photoinactivation (TFI) is the time (s) for oxygen evolution to be come completely inhibited during continuous actinic illumination. [d]The values for PSII abundance have been normalized to a chlorophyll/PSII ration of 390. [e]Stable rate of oxygen evolution. [f]This measurement was only performed once. [g]The observed rate of oxygen evolution showed evidence of photoinactivation. The data are an average of two to five independent measurements and were reproducible within 15% of the average unless indicated otherwise.

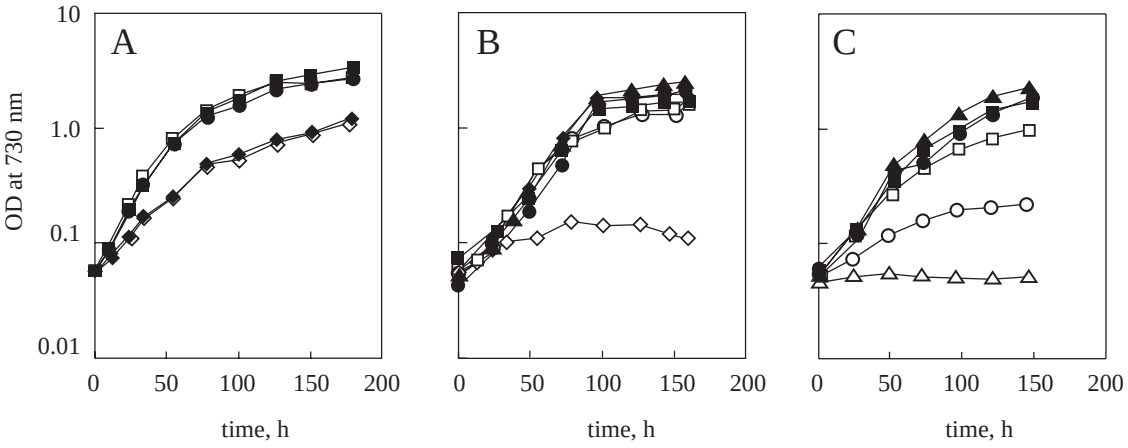

Fig. 4. Photoautotrophic growth curves of *Synechocystis* sp. PCC 6803 strains as measured by the optical density at 730 nm in BG-11. A. Open symbols are with 480 μM sodium chloride and closed symbols are with 480 μM sodium bromide. Control (circles), ΔT436 (squares) and ΔF430 (diamonds). B. When present: sodium chloride was at 480 μM, glucose was at 5 mM and diuron was at 20 μM. Control without chloride (open circles); ΔT436 without chloride (open squares); ΔF430 without chloride (open diamonds); control with chloride, glucose and diuron (closed circles); control without chloride but with glucose and diuron (closed triangles); ΔT436 without chloride but with glucose and diuron (closed squares) and ΔF430 without chloride but with glucose and diuron (closed diamonds). C. Open symbols are for the ΔF430 strains and closed symbols are for the ΔT436 strains. Strains without PSII-O (circles), strains without PSII-U (squares) and strains without PSII-V (triangles).

previously mutated Glu-364 and Asp-331 and had not observed any deleterious effects. In contrast, substitutions at Glu-364 were found to be lethal when combined with a *psbV* deletion that removed PSII-V (Morgan *et al*., 1998). Therefore we introduced substitutions, in the presence or absence of PSII-V, at Pheo-430, Asp-331, Asp-333 and Thr-436; however, these mutants possessed a similar phenotype to the corresponding control strains. However, when we extended our characterizations to include the four mutants, ΔF430, ΔD430, ΔD433 and ΔT436, we found that the ΔF430 strain possessed a phenotype that was similar to the Δ(G429-T436) mutant. This included the loss of photoautotrophic growth upon the removal of the PSII-V protein and the introduction of an altered chloride dependence on PSII activity. The Phe-430 residue was found in all CP47 sequences except in *Guillardia theta*, where a Leu is at this position. We examined the effect of replacing Pheo-430 with a Leu in *Synechocystis* sp. PCC 6803 and found that the phenotype remained similar to that of the control strain. A Pheo residue is also conserved at position 432 in PSII-V type photosystems that can be substituted by a Leu in PSII-P type PSII (see Fig. 3). However, at this time no mutations have been introduced at this position. Finally, since Phe-430 is a hydrophobic amino acid, the observed chloride dependence in the ΔF430 strain is likely to arise from disruption of a specific protein-protein interaction, and it is noteworthy that there was no effect when the neighboring Asp-431 was deleted. Interestingly, a similar chloride dependence was introduced when Phe-363 was mutated in Clarke and Eaton-Rye (1999). However, an understanding of the impact of these mutations on PSII function will require additional experimental and/or structural details.

Acknowledgement

This work was supported by a New Zealand Marsden grant (UOO 521) and an Otago University Research grant to JJER. We thank Professor M. Ikeuchi for the antibodies.

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