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Rapid report

Introduction of the ^{305}Arg→^{305}Ser mutation in the large extrinsic loop E of the CP43 protein of *Synechocystis* sp. PCC 6803 leads to the loss of cytochrome c_{550} binding to Photosystem II☆

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Abstract

CP43, a component of Photosystem II (PSII) in higher plants, algae and cyanobacteria, is encoded by the *psbC* gene. Previous work demonstrated that alteration of an arginine residue occurring at position 305 to serine produced a strain (R305S) with altered PSII characteristics including lower oxygen-evolving activity, fewer assembled reaction centers, higher sensitivity to photoinactivation, etc. [Biochemistry 38 (1999) 1582]. Additionally, it was determined that the mutant exhibited an enhanced stability of its S_2 state. Recently, we observed a significant chloride effect under chloride-limiting conditions. The mutant essentially lost the ability to grow photoautotrophically, assembled fewer fully functional PS II reaction centers and exhibited a very low rate of oxygen evolution. Thus, the observed phenotype of this mutation is very similar to that observed for the ΔpsbV mutant, which lacks cytochrome c_{550} (Biochemistry 37 (1998) 1551). A His-tagged version of the R305S mutant was produced to facilitate the isolation of PSII particles. These particles were analyzed for the presence of cytochrome c_{550}. Reduced minus oxidized difference spectroscopy and chemiluminescence examination of Western blots indicated that cytochrome c_{550} was absent in these PSII particles. Whole cell extracts from the R305S mutant, however, contained a similar amount of cytochrome c_{550} to that observed in the control strain. These results indicate that the mutation R305S in CP43 prevents the strong association of cytochrome c_{550} with the PSII core complex. We hypothesize that this residue is involved in the formation of the binding domain for the cytochrome.

Keywords: CP43; Oxygen evolution; Photosystem II; Chloride; Cytochrome c_{550}

The light-driven oxidation of water to molecular oxygen and concomitant reduction of plastoquinone to plastoquinol is catalyzed by the Photosystem II (PSII) complex. PSII consists of both intrinsic polypeptide subunits embedded within the thylakoid membrane and extrinsic components exposed to the thylakoid lumen. The intrinsic polypeptide components necessary for the formation of a PSII complex competent in oxygen evolution are CP47, CP43, D1, D2, the α and β subunits of cytochrome b_{559}, the 4 kDa *psb*I gene product and a number of other low molecular mass components. In higher plants, three extrinsic proteins with apparent molecular masses of 33 (manganese-stabilizing protein, MSP), 24 and 17 kDa are required for maximal rates of oxygen evolution under physiological ionic conditions. The 24 and 17 kDa proteins are absent in cyanobacteria, but appear to be functionally replaced by cytochrome c_{550} and the 12 kDa protein [1]. In the absence of the extrinsic proteins, PSII complexes retain the ability to evolve oxygen, but at significantly reduced rates [2,3]. This ability to evolve oxygen is dependent upon the presence of high, nonphysiological, concentrations of calcium and chloride.

The product of the *psb*C gene, CP43 is an integral thylakoid protein and component of the proximal antenna of PSII [4]. Hydropathy analysis predicts that CP43 contains six transmembrane alpha helices [5]. This has been con-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCBQ, 2,6-dichloro-*p*-benzoquinone; HA, hydroxylamine; kb, kilobase pair(s); kDa, kilodaltons; LDS, lithium dodecylsulfate; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSP, manganese-stabilizing protein; PBP, phycobiliprotein; PSII, Photosystem II; PCR, polymerase chain reaction

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firmed by the recent 3.8 Å resolution crystal structure of PS II from *Synechococcus elongatus* [6]. In addition to the six membrane-spanning regions, CP43 also contains five hydrophilic loops that connect the membrane-spanning domains. One of these loops, the large extrinsic loop E, spans amino acid residues ^{278}Asn–^{410}Trp and is located between the fifth and sixth membrane-spanning helices. This large extrinsic loop is exposed to the lumenal side of the thylakoid membrane [7]. While CP43 functions as a proximal chlorophyll-*a* light-harvesting antenna for PSII, it has become increasingly clear that CP43 also plays a role in the stable assembly of PS II and in the oxygen-evolving process [8]. *Chlamydomonas* mutants bearing alterations in *psb*C that affect either the synthesis or stability of CP43 are deficient in PSII activity [9]. In these mutants, the levels of other PSII core proteins within the thylakoid are severely reduced. Deletion of *psb*C in *Synechocystis* produces mutants that accumulate PSII core complexes to only 10% of wild type levels and do not grow photoautotrophically or evolve oxygen [10,11]. Eight short deletions were introduced within the large extrinsic loop E of CP43 in *Synechocystis* by Kuhn and Vermaas [12]. Significantly, all resulting mutants showed complete loss of photoautotrophic growth and the ability to evolve oxygen. These mutants also contained decreased levels of the PSII reaction center proteins D1, D2 and CP47. Site-directed mutagenesis of an arginine residue at position 305 in the large extrinsic loop E of CP43 produced a mutant that exhibited decreased rates of steady state oxygen evolution, enhanced rates of photoinactivation, an increased fluorescence rise time and decreased numbers of fully functional PSII centers [13]. These data collectively suggest that CP43 is essential for normal PSII assembly and function.

Cytochrome c_{550} is an unusual low potential cytochrome (-250 mV) associated with the oxygen-evolving complex of PSII in cyanobacteria [1,14] and is encoded by the *psb*V gene. It appears to regulate the efficiency of the $S_1 \rightarrow S_2$ and/or the $S_2 \rightarrow S_3$ state transitions. Mutants which lack the cytochrome (ΔpsbV strains) exhibit a stabilization of the S_2 state and cannot grow photoautotrophically under chloride- or calcium-limiting conditions [15]. Cytochrome c_{550} may function in a manner similar to that of the 24 kDa protein in higher plant PSII.

High-resolution crystal structures for isolated cytochrome c_{550} are available [16,17] and indicate that it is a bis-histidinyl monoheme cytochrome. Examination of the Zouni crystal structure of PSII [6] indicates that electron density assigned to the MSP appears to be positioned over the assigned D2/CP47 side of the reaction center while cytochrome c_{550} appears to be positioned over the assigned D1/CP43 region. At the current resolution, however, it is difficult to unambiguously trace the backbone of the intrinsic membrane proteins to identify the domains of interaction between the intrinsic and extrinsic components of the photosystem. Site-directed mutagenesis studies have indicated that a binding domain for the MSP exists on CP47 at arginyl residues 384 and 385 [18–20]. No similar studies identifying the binding domains for cytochrome c_{550} or the 12 kDa proteins have been performed.

In this report, we demonstrate that while cell homogenates of the R305S mutant strain contain normal amounts of cytochrome c_{550}, isolated PSII particles contain no detectable amounts of the cytochrome. We hypothesize that ^{305}R participates in the formation of a binding domain for this extrinsic component of PSII.

The construction of the kanamycin-resistant control strain and the R305S mutant was described previously [13,21]. A His-tagged version of the R305S mutant was constructed as described below for facilitating the isolation of PSII particles from the R305S mutant. A 1.3 kb fragment from the HT3-A strain of *Synechocystis*, containing a 6-histidine tag at the 3′ terminus of the coding sequence of CP47, was amplified using polymerase chain reaction (PCR). Amplification was done directly from cells using colony PCR. A single colony large enough to see with the unaided eye (approximately 0.25 mm) was harvested and added to a 0.5 ml reaction tube. The remainder of the PCR reaction consisted of the following: 4 pmol of the forward and reverse primers (5′-GATAGCCAGTTAGCAGAA-3′ and 5′-GGAGCATAA-TAGTG-CAAC-3′); 4 μl of 50 mM $MgCl_2$; 8 μl of 1.0 mM dNTP's; 10 μl of 10 × PCR buffer (GibcoBRL); 0.5 μl of 5 U/μl Taq DNA polymerase (GibcoBRL); and dH_2O to a final volume of 100 μl. The thermal cycling routine was as follows: first cycle; 12 min at 94 °C (an additional 10 min at denaturing temperature is required to lyse the cells as well as to denature proteases), 1 min at 52 °C, 3 min at 72 °C. Each of the next 28 cycles consisted of: 45 s at 94 °C, 40 s at 52 °C and 3 min at 72 °C. The last cycle included incubation at 72 °C for 5 min then proceeding directly to 4 °C. The PCR product was then ligated into the pGEM-T vector (Promega), following the kit instructions. A 2.0 kb spectinomycin-resistance cartridge was blunt end-ligated into an *Nco*I restriction site located 367 bp downstream of the 3′ end of the CP47 coding region. The resulting plasmid was used to transform both the control and R305S strains in order to introduce a histidine tag that was used for the purpose of purifying PSII particles. The genomic DNA of spectinomycin-resistant transformants was sequenced [13] in order to confirm the presence of the histidine tag.

Control and mutant *Synechocystis* sp. PCC 6803 were grown in liquid BG-11 media [25] supplemented with glucose (5 mM), the appropriate antibiotics (10 μg/ml) and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) at 30 °C and at a light intensity of 25 μmol photons m^{-2} s^{-1}. His-tagged PSII particles were isolated by metal-affinity chromatography as described by Frankel et al. [22] on either cobalt or nickel columns. Difference spectroscopy for the detection of cytochrome c_{550} and cytochrome b_{559} was performed essentially as described by Kashino et al. [23]. Samples were suspended at 20 μg/ml chl in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH, pH 6.5, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 50 mM NaCl, 0.04% β-D-dodecyl maltoside and 25% glycerol. After collection of a baseline on a Varian 100 dual

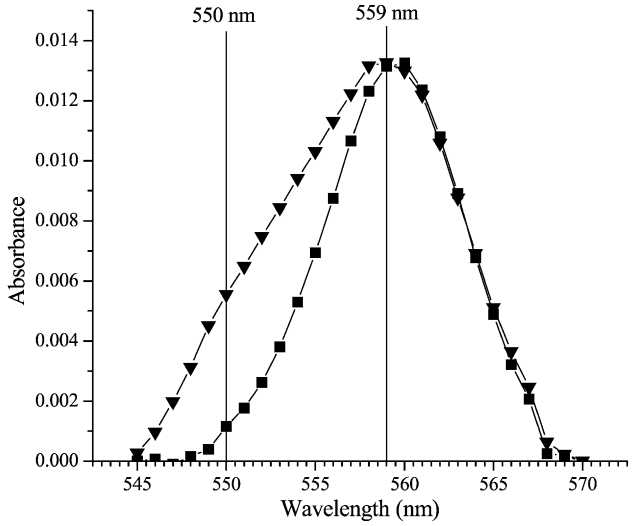

Fig. 1. Dithionite-reduced minus ferricyanide-oxidized difference spectra of control and R305S His-tagged PS II particles. The spectral baseline was linearly adjusted between local absorbance minima at 545 and 570 nm. The spectrum of the control particles is shown as triangles while that of the R305S particles is shown as squares.

beam spectrophotometer, the samples were either reduced with a few crystals of sodium dithionite or oxidized with a few crystals of potassium ferricyanide. After incubation in the dark for 10 min, reduced minus oxidized difference spectra were then collected. PSII particles were also subjected to electrophoresis on lithium dodecylsulfate (LDS)-polyacrylamide gels and Western blotted onto Immobilon PVDF membranes (Millipore Corp.). Cytochromes were detected on the blots by chemiluminescence. The blots were incubated for 5 min with the chemiluminescence substrates (SuperSignal West Dura Extended Duration Substrate, Pierce-Endogen Co.) as per the manufacturer's instructions. BioMax ML X-ray film (Kodak Co.) was then exposed to the blots and incubated for 30 s to 5 min and the film subsequently developed. This technique yields a strong reproducible signal for the detection of *c*-type cytochromes and appears to be much more sensitive than staining polyacrylamide gels with either 3,3′,5,5′-tetramethylbenzidine or 3,3′-diaminobenzidine. Following the detection of the chemiluminescent signal, the blots were stained with Coomassie blue.

The phenotype of the R305S mutant under chloride-sufficient conditions and chloride-limiting conditions will be described elsewhere (A. Young, M. McChargue, L.K. Frankel, T.M. Bricker, C. Putnam-Evans, submitted for publication). To study the possible effects of the mutation on PSII assembly, the PSII complexes of both mutant and control strains grown in chloride-depleted media were isolated utilizing a 6-His tag present on the 3′ terminus of the CP47 protein. These PSII particles evolved oxygen and exhibited an absorption spectrum (data not shown) very similar to that seen in the CP47 His-tagged PSII preparation described previously [24]. Immunoblots of the isolated PSII core particles demonstrated that for control and R305S cells, there was no detectable loss of any of the PSII proteins that were investigated, including CP43, CP47, D1 and the MSP. These results are similar to those obtained previously using isolated thylakoids from control and R305S strains grown in chloride-sufficient media [13].

The PSII particles were also probed for the presence of cytochrome c_{550}. Fig. 1 shows reduced minus oxidized difference spectra for the control and R305S His-tagged PSII particles. The control particles exhibited an asymmetric peak with a maximum absorbance at 559 nm with a pronounced shoulder at about 550 nm. These peaks arise from cytochrome b_{559} and c_{550}, respectively [24]. The R305S PSII particles, however, exhibited a highly symmetrical peak with no apparent shoulder at 550 nm and an absorption maximum of 559.5 nm. This result indicated that the mutant particles contained little or no cytochrome c_{550}. To further test for the presence of this cytochrome, Western blots of the wild type and mutant particles were probed for *c*-type cytochromes

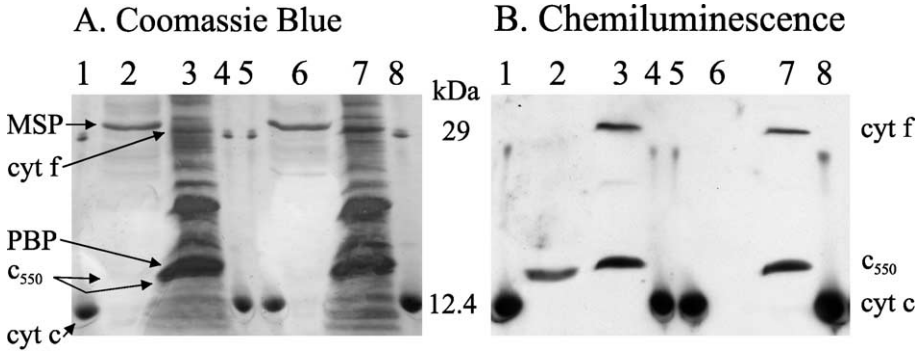

Fig. 2. Western blot analysis of whole cell extracts and His-tagged PS II particles from the control strain and the R305S mutant either stained with Coomassie blue (Panel A) or imaged by chemiluminescence (Panel B). Lanes 1, 4, 5 and 8 are standard proteins. Lanes 2 and 6 are His-tagged PS II particles from the control strain and R305S mutant, respectively. Lanes 3 and 7 are whole cell extracts of the control strain and R305S mutant, respectively. Specific components are identified on the left and right sides of the figure while molecular weights of the standards are indicated between the two panels. A major phycobiliprotein (PBP), which was identified by its blue color on the unstained gel, comigrates with cytochrome c_{550} in the cell extract lanes (lanes 3 and 7). Horse heart cytochrome *c*, a component of our standard protein mixture, yields the chemiluminescent band observed in the standard protein lanes (lanes 1, 4, 5 and 8) in Panel B.

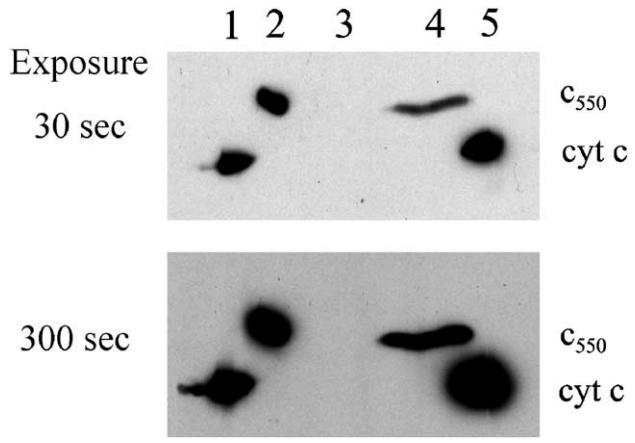

Fig. 3. Chemiluminescent analysis of control and R305S His-tagged particles. Lanes 1 and 5, standard proteins. Lanes 2 and 4, two independent preparations of control His-tagged particles. Lane 3, R305S His-tagged particles. The X-ray film was exposed for either 30 or 300 s. No cytochrome c_{550} was observed even with the 10× overexposure.

using a chemiluminescent procedure. *c*-type cytochromes exhibit peroxidase activity which allows their detection by 3,3′,5,5′ tetramethylbenzidine, 2,2′-diaminobenzidine or chemiluminescence. Fig. 2 shows the results of this analysis. Whole cell extracts of both the control and R305S strains exhibit chemiluminescent bands (Fig. 2B) at apparent molecular masses of 32 and 16 kDa (lanes 3 and 7), which correspond to cytochrome *f* and cytochrome c_{550}, respectively. Generally, *b*-type cytochromes do not survive denaturing electrophoresis. Consequently, no band arising from cytochrome b_{559} was observed. Also, the standard protein lanes (1, 4, 5, and 8) contained horse heart cytochrome *c* (ca. 100 ng), which yields a chemiluminescent band. PSII particles from the control strain (lane 2) exhibit only a single chemiluminescent band at an apparent molecular mass of 16 kDa, which corresponds to the presence of cytochrome c_{550}. Cytochrome *f* is, of course, highly depleted in the PSII preparation. Interestingly, the mutant PSII preparation (lane 6) exhibited no detectable chemiluminescent bands. This result indicates the absence of any chemiluminescent-detectable cytochrome c_{550} in PSII particles isolated from the R305S mutant strain. The Western blot was then stained with Coomassie blue and is shown in Fig. 2A. The cellular extracts contain many protein components, the vast majority of which are depleted in the lanes containing the His-tagged PSII particles.

To further evaluate the presence of cytochrome c_{550} in the His-tagged PSII particles, Western blots were overexposed in an effort to detect the possible presence of any small amount of cytochrome c_{550} that might be associated with the particles (Fig. 3). After a 30-s exposure, chemiluminescent bands arising from cytochrome c_{550} are clearly present in the lanes containing two independent control strain PSII particle preparations (lanes 2 and 4). No chemiluminescent band was observed in the lane containing R305S PSII particles (lane 3). A 10-fold overexposure of the Western blot yielded identical results; no cytochrome c_{550} was detected.

At least two hypotheses can be formulated which can explain these results. First, the introduction of the $^{305}R \rightarrow {}^{305}S$ mutation in the large extrinsic loop of CP43 could lead to a global alteration in the structure of CP43 which prevents cytochrome c_{550} from binding to other components in the photosystem. We feel that this is unlikely. Even short deletions in the large extrinsic loop of CP43 lead to loss of the protein and failure to assemble functional PSII reaction centers [12]. If a global disruption of the structure of the extrinsic loop of CP43 was introduced by the point mutation, we would expect that the phenotype of the R305S mutant would be substantially more severe than we have observed [13]. Under normal growth conditions, R305S exhibits a relatively mild phenotype and is capable of assembling substantial numbers of reaction centers that can evolve oxygen. A second hypothesis is that ^{305}R participates in the formation of a binding domain for cytochrome c_{550}. We favor this latter hypothesis. The presence of c_{550} in the whole cell extracts from the mutant indicates that this component is present but that it cannot associate strongly with the photosystem. It should be noted that our results do not preclude the possibility that other binding domains contribute to the overall association of the cytochrome to the photosystem. Our results indicate that the alteration of ^{305}R to a neutral residue is sufficient to prevent functional binding of the cytochrome to PSII particles. Since particle preparation involves disruption of the thylakoid membrane, treatment with a relatively high concentration of detergent and column chromatography, it is formally possible that the cytochrome may associate weakly to PSII in vivo in the R305S strain via other binding domains, but that the rigors of particle isolation preclude this association from persisting in vitro. We are currently investigating the binding kinetics of cytochrome c_{550} to both control and R305S His-tagged particles to examine this possibility.

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