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The contribution of D2-Arg265 to the molecular architecture of the quinone-Fe-bicarbonate acceptor complex of photosystem II

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ABSTRACT

In Photosystem II (PS II) the D2 and D1 proteins provide binding sites for the primary (Q_A) and secondary (Q_B) plastoquinone electron acceptors, respectively. A non-heme iron is located between Q_A and Q_B that is coordinated by a bicarbonate ligand and two His residues from D1 (D1-His215 and D1-His272) and two His residues from D2 (D2-His214 and D2-His268). The symmetry of the quinone-Fe-acceptor complex extends to D1-Arg269, which has hydrogen bonds to D1-His272, and D2-Arg265 which has hydrogen bonds to D2-His268. We have examined the role of D2-Arg265 by creating the R265A and R265D mutants in the cyanobacterium *Synechocystis* sp. PCC 6803. Both mutants exhibited normal photoautotrophic growth, but showed a reduction in oxygen evolution in the presence of the PS II-specific electron acceptor 2,5-dimethyl-1,4-benzoquinone (DMBQ). Chlorophyll *a* fluorescence induction and decay kinetics were also inhibited in the presence of DMBQ and, in the presence of the native quinone, revealed slowed Q_A^- to Q_B electron transfer, together with impaired exchange between the Q_B -binding site and the plastoquinone pool. Addition of formate further inhibited electron transfer, consistent with weakened bicarbonate binding in the mutants, and thermoluminescence measurements revealed a decreased redox gap between Q_A and Q_B . Additionally, both mutants displayed heightened sensitivity to high light. These findings demonstrate that D2-Arg265 is important for stability of the acceptor side, bicarbonate-dependent electron transfer, and an optimal Q_B -binding site. All of our results are also consistent with the architecture of the quinone-Fe-bicarbonate complex supporting photoprotection and regulatory roles that are unique to oxygenic photosynthesis.

1. Introduction

Photosystem II (PS II) is the thylakoid membrane complex responsible for light-driven oxidation of water in oxygenic photosynthesis [1–3]. Cyanobacteria have been extensively used as model systems to study structure-function relationships in PS II [4–7]. In cyanobacteria the mature PS II complex is dimeric and has a total mass of approximately 700 kDa, where each monomer consists of around 20 subunits. Within each monomer, the reaction center proteins D1 and D2 are

responsible for coordinating most redox-active cofactors, with the D1 protein providing ligands for the Mn₄CaO₅ oxygen-evolving complex (OEC). D1 and D2 form a heterodimer flanked by two core antenna chlorophyll *a*-binding proteins CP43 and CP47, with CP43 also contributing to the OEC binding site [8,9]. Surrounding the CP43-D1-D2-CP47 core are 13 low-molecular-weight (LMW) proteins, and three to four extrinsic proteins are located on the luminal side of the complex where they contribute to the stabilization of the OEC and participate in proton release and substrate water access [5,7]. During water splitting,

Abbreviations: Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; BG-11, blue-green 11 medium; BN-PAGE, blue-native polyacrylamide gel electrophoresis; CP43, 43 kDa chlorophyll *a*-binding core antenna protein; CP47, 47 kDa chlorophyll *a*-binding core antenna protein; Cryo-EM, Cryo-electron microscopy; D1, the *psbA*-encoded reaction center protein subunit; D2, the *psbD*-encoded reaction center protein subunit; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ, 2,5-dimethyl-1,4-benzoquinone; F, fluorescence level; F_m , maximum fluorescence level; F_o , initial fluorescence level; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LMW, low-molecular-weight; OEC, oxygen-evolving complex; O, J, I and P, initial fluorescence level, J inflection, I inflection and peak of the fluorescence induction curve; P680, the reaction center chlorophyll pigments; P680*, the excited state of P680; PCC, Pasteur Culture Collection; PS II, Photosystem II; Q_A , primary plastoquinone electron acceptor of PS II; Q_B , secondary plastoquinone electron acceptor of PS II; ROS, reactive oxygen species; S states, the five, S_0 ... S_4 , oxidation states of the oxygen-evolving complex following single-turnover flashes applied to dark-adapted cells.

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the OEC progresses through a series of oxidation states (S_0 to S_4) providing electrons for PS II and generating molecular oxygen [10–12].

In cyanobacteria, the assembly of PS II has been shown to follow a modular process, where the initial formation of the reaction center complex arises from the association of pre-assembled D1 and D2 modules [13,14]. Subsequently, CP47 binds to form the RC47 intermediate, followed by the integration of CP43, the light-driven assembly of the OEC, and the attachment of the extrinsic subunits [15,16]. In the final steps, PS II dimerization occurs and the complex associates with the peripheral phycobilisome antenna system. This assembly process is facilitated by various assembly factors, which are transiently involved but absent from the mature PS II complex [16].

Despite its essential role in oxygenic photosynthesis, PS II is particularly vulnerable to damage resulting from reactive oxygen species (ROS) generated during water splitting and electron transfer processes [17–19]. Under stressful conditions, such as excessive light exposure, photoinhibition occurs when the damage rates exceed the repair capacity of PS II, leading to reduced photosynthetic efficiency and impaired electron transport [20,21].

The electron transfer process in PS II is initiated when light energy is received, via absorption in the antenna system, by the reaction center chlorophylls (in P680) resulting in charge separation and the generation of $P680^+$ [22]. The excited $P680^*$ donates an electron to pheophytin, which then passes the electron to the primary plastoquinone (PQ) acceptor, Q_A , located on the D2 protein. Subsequently, the electron is transferred to the secondary PQ acceptor Q_B , which is bound to the D1 protein. Q_B undergoes two reduction and protonation steps, resulting in the formation of plastoquinol (Q_BH_2 or PQH_2) [23]. After its formation, Q_BH_2 dissociates from the Q_B -binding site and is replaced by oxidized PQ from the thylakoid membrane pool [24].

A non-heme iron (NHI) is located between Q_A and Q_B . This iron is coordinated by four histidine residues – two from the D1 protein (His215 and His272) and two from the D2 protein (His214 and His268), as well as a bidentate bicarbonate ligand (Fig. 1) [4,9,25,26]. The bound bicarbonate facilitates electron transfer from reduced Q_A to Q_B [27,28] and contributes to stabilizing the electron acceptor side of PS II [15]. In

addition, bicarbonate-binding tunes the redox potential of the Q_A/Q_A^- couple with bicarbonate release suggested to protect against photo-damage in photochemically closed reaction centers, by downregulating $^1O_2$ production [29,30].

Both the D1 and D2 proteins comprise five membrane-spanning helices (A to E) and both contain a conserved arginine cap associated with helix E; D1-Arg269 and D2-Arg265, respectively. Previous studies on D1-Arg269 in *Chlamydomonas reinhardtii* demonstrated that its replacement with glycine severely compromised PS II stability, rendering cells incapable of photoautotrophic growth or oxygen evolution [31,32]. This mutation also disrupted both the acceptor and donor side electron transfers, altered the Q_B binding site, decreased the number of assembled PS II centers, and increased the sensitivity of cells to formate, an inhibitor of bicarbonate binding [31,32]. Despite these insights into the role of D1-Arg269, the functional significance of its counterpart, D2-Arg265, remains largely unexplored. A brief mention in a review by Diner et al. [33] suggested that mutations at D2-Arg265 led to a 40-fold decrease in bicarbonate binding affinity and altered Q_A reoxidation kinetics; however, no further studies have been conducted on these reactions.

We have used the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) to study D2-Arg265 (Fig. S1). Structural studies in *Synechocystis* 6803 placed D2-Arg265 3.3 Å from D2-His268, one of the axial ligands to the NHI, and 8.7 Å from the bicarbonate ligand [7]. Similarly, D1-Arg269 is located 3.3 Å from D1-His272, indicating a conserved spatial arrangement of these arginine residues relative to the histidine ligands coordinating the NHI. D2-Arg265 may influence bicarbonate binding through a hydrogen bond network involving the nearby residues. Notably, a recent study on D2-Lys264, showed that perturbations at this position disrupted bicarbonate binding, leading to impaired PS II function and assembly [34]. Given the position of D2-Arg265 in the vicinity of residues involved in coordinating the NHI and in bicarbonate binding, we examined the impact of introducing mutations at D2-Arg265. Our data suggest that D2-Arg265 contributes to stabilizing the quinone-Fe-acceptor complex and hence contributes to efficient forward electron transfer from the reduced Q_A to Q_B .

2. Materials and methods

2.1. Strains and culture conditions

The glucose-tolerant *Synechocystis* sp. PCC 6803 GT-O1 substrain was used in this study [35,36]. In *Synechocystis* 6803, the D2 protein is encoded by two genes, *psbDI* and *psbDII*, with *psbDI* located within the *psbDI:psbC* operon [37,38].

Two mutant strains (R265A and R265D) were generated by introducing modified *psbDI:psbC* operons into a deletion strain lacking both *psbDII* and the *psbDI:psbC* operon [39]. In this deletion strain, *psbDII* was replaced with a kanamycin-resistance cassette, while the *psbDI:psbC* operon was substituted with a chloramphenicol-resistance cassette. To facilitate the selection for successful incorporation of the modified operon, a spectinomycin-resistance cassette was inserted downstream of *psbC*. Mutations in the D2 protein were introduced using the Quik-Change II site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA) with primers listed in Table S1; however, the control strain retained the wild-type *psbDI* sequence. The genotype and the segregation of all the strains were confirmed via colony PCR and Sanger sequencing.

Strains were maintained on BG-11 agar plates supplemented with 5 mM glucose, 20 μM atrazine, 10 mM TES-NaOH (pH 8.2), and 0.3 % sodium thiosulfate. Liquid cultures were grown mixotrophically in BG-11 medium containing 5 mM glucose, with appropriate antibiotics: chloramphenicol (15 μg mL⁻¹), kanamycin (25 μg mL⁻¹), and spectinomycin (25 μg mL⁻¹). Cultures were illuminated with 30 μmol photons m⁻² s⁻¹ provided by two Philips HPL-N 250 W metal halide bulbs at 30 °C. Photoautotrophic growth assays were conducted under identical

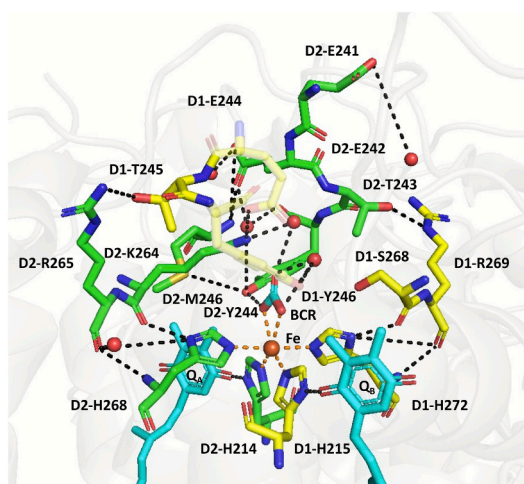


Fig. 1. Location of D2-Arg265 and D1-Arg269 in proximity to the non-heme iron and bicarbonate-binding environment of the acceptor side of PS II. BCR represents bicarbonate, Fe is the non-heme iron and red spheres indicate water molecules. Q_A and Q_B are shown in light blue. Oxygen atoms are shown in red, nitrogen atoms are shown in blue, and the sulfur atom in Met246 is shown in yellow orange. D1 residues are in yellow and D2 residues are in green. Dashed black lines indicate putative hydrogen bonds or salt bridge interactions. The coordination bonds to the non-heme iron are shown in orange. The figure was generated using PyMOL and PDB 7N8O. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

light and temperature conditions but without glucose [40].

2.2. Oxygen evolution and photodamage assays

Oxygen evolution rates were measured using a Clark-type oxygen electrode (Hansatech, King's Lynn, UK) at 30 °C. Samples were adjusted to 10 µg mL⁻¹ chlorophyll *a* and PS II-specific electron transfer was measured in the presence of 0.2 mM 2,5-dimethyl-1,4-benzoquinone (DMBQ) supplemented with 1 mM potassium ferricyanide (K₃Fe(CN)₆). Whole chain electron transfer was measured in the presence of 15 mM sodium bicarbonate. When present, sodium formate was added to a final concentration of 25 mM. Saturating light (2000 µmol photons m⁻² s⁻¹) was provided by an FLS1 light system (Hansatech, UK) passed through a Melis Griot OG 590 sharp cutoff red glass filter.

To assess photodamage and PS II repair, samples were subjected to high-light stress (2000 µmol photons m⁻² s⁻¹ for 45 min) using an Ektalite slide projector (Kodak, Rochester, NY, USA). Cultures were then transferred back to the growth-light conditions (30 µmol photons m⁻² s⁻²) for 135 min to allow for PS II recovery. Measurements were taken every 15 min over a 180-min period, using DMBQ with K₃Fe(CN)₆ or sodium bicarbonate to support electron transport. Cells were maintained at 30 °C throughout the assay.

2.3. Low-temperature (77 K) fluorescence emission spectra

Fluorescence emission spectra at 77 K were recorded using a modified MPF-3L fluorescence spectrometer (PerkinElmer, Waltham, MA, USA) with a custom-built Dewar system. Samples containing 5 µg mL⁻¹ chlorophyll *a* were snap-frozen, and spectra were collected following excitation at 440 nm or 580 nm. Data were normalized to the PS I emission maximum at 725 nm [41,42].

2.4. Measurement of variable chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence induction and decay kinetics were measured using a FL-3500 fluorometer (PSI Instruments, Brno, Czech Republic). All measurements were made at a chlorophyll *a* concentration of 5 µg mL⁻¹. For fluorescence induction experiments, cells were dark-adapted for 5 min, then given a constant blue (455 nm) actinic light and blue (455 nm) measuring flashes. For fluorescence decay measurements, samples were dark-adapted for 5 min before being exposed to either a single-turnover actinic 455 nm flash or a train of saturating flashes spaced at 200 ms intervals (5 Hz). Fluorescence decay was monitored by giving blue (455 nm) measuring flashes [41,42]. Kinetic analyses were performed following the methods of Vass et al. [43]. When present, sodium bicarbonate was at 15 mM, sodium formate at 25 mM, and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) at 40 µM.

2.5. Thermoluminescence measurements

Thermoluminescence (TL) was measured according to [44]. Samples containing 30 µg chlorophyll *a* were exposed to a single actinic flash at -10 °C, then cooled to -40 °C. TL signals were recorded as the sample was heated at 20 °C min⁻¹ to 80 °C. When used, 60 µM DCMU was added before the actinic flash.

2.6. Isolation of thylakoid membranes

Thylakoid membranes were isolated from cultures grown to an OD₇₅₀ of 0.8. Cells were harvested and washed in a buffer containing 1 mM 6-amino caproic acid, 2 mM benzamidine, 20 mM CaCl₂, 50 mM HEPES-NaOH (pH 7.5), 10 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride. Cells were resuspended in disruption buffer, composed of the wash buffer that additionally contained 1 M betaine monohydrate and 800 mM sorbitol, and, then, transferred to tubes containing 0.1 mm zirconia beads (BioSpec Products, Bartlesville, OK, USA). Mechanical

cell breakage was performed using a bead beater (BioSpec Products) for five cycles (25 s at 4800 rpm, followed, per cycle, by 5 min incubation on ice). After disruption, intact cells and debris were removed by centrifugation at 8000 ×g for 5 min. Thylakoid membranes were collected via centrifugation at 25,000 ×g for 1 h at 4 °C, then resuspended in the disruption buffer and centrifuged again at 25,000 ×g for 25 min at 4 °C. Purified thylakoid membranes were snap-frozen in liquid nitrogen and stored at -80 °C in a buffer containing 25 mM Bis-Tris-HCl (pH 7.0), 20 % glycerol, and 0.25 mg mL⁻¹ Pefabloc (Sigma-Aldrich, St. Louis, MO, USA).

2.7. Blue-native polyacrylamide gel electrophoresis and western blotting

Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was performed according to Fagerlund et al. [45]. Thylakoid samples containing 1.2 µg chlorophyll *a* were loaded onto 4–16 % Bis-Tris gradient gels (Life Technologies, Carlsbad, CA, USA) and subjected to electrophoresis at 4 °C.

Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and probed with D1 (AS05 084), D2 (AS06 146), and CP43 (AS11 1787)-specific antibodies (Agrisera, Vännäs, Sweden). Anti-rabbit IgG peroxidase (Sigma-Aldrich) was used as the secondary antibody, and protein visualization was performed using enhanced chemiluminescence detection.

3. Results

3.1. Mutations targeting D2-Arg265 do not prevent PS II assembly or photoautotrophic growth

To initially evaluate the effects of mutations at D2-Arg265 on PS II the photoautotrophic growth rates of the mutant strains were assessed (Fig. 2A). Both the R265A and R265D mutants demonstrated the ability to grow photoautotrophically, exhibiting doubling times of 15 h, which were comparable to the control strain. This suggests that substitutions at D2-Arg265 do not severely impair the overall viability of cells under photoautotrophic conditions. Subsequently, the mutants were examined using 77 K low-temperature fluorescence emission spectroscopy. Following excitation at 440 nm, fluorescence peaks were observed at 685 nm, corresponding to emission from CP43 and CP47 in unassembled PS II, and 695 nm from CP47 in assembled PS II [46]. In addition, PS I emission was observed at 725 nm [47]. In both the R265A and R265D strains, there was a reduction in fluorescence yield at 685 nm and 695 nm, suggesting a decrease in the PS II to PS I ratio (Fig. 2B). This indicates that while PS II remains functionally present in these strains, its relative abundance is lower compared to the control.

Fluorescence emission following 580 nm excitation was also examined. Excitation at 580 nm primarily excites phycobilins in the phycobilisome, and the resulting spectra revealed minor reductions in fluorescence peaks at 645 nm and 665 nm in R265A and R265D cells (Fig. 2C). These peaks correspond to phycocyanin and allophycocyanin emission, respectively, suggesting slight alterations in energy transfer from the phycobilins to the chlorophyll *a* in the photosystems. Additionally, a slight increase in the 685 nm peak was observed in both mutants, which could indicate a degree of phycobilisome uncoupling or altered energy distribution between PS I and PS II.

PS II assembly was also examined through BN-PAGE, followed by western blot analysis (Fig. 3). Both the R265A and R265D mutants exhibited levels of PS II complexes similar to those in the control strain. However, there was a slight accumulation of the RC47 preassembly complex and the PS II monomer in both the mutants. This accumulation suggests that while the mutations at D2-Arg265 do not severely disrupt PS II assembly, they may cause minor disruptions in the incorporation of CP43 into the RC47 complex and potentially modify PS II dimer stability.

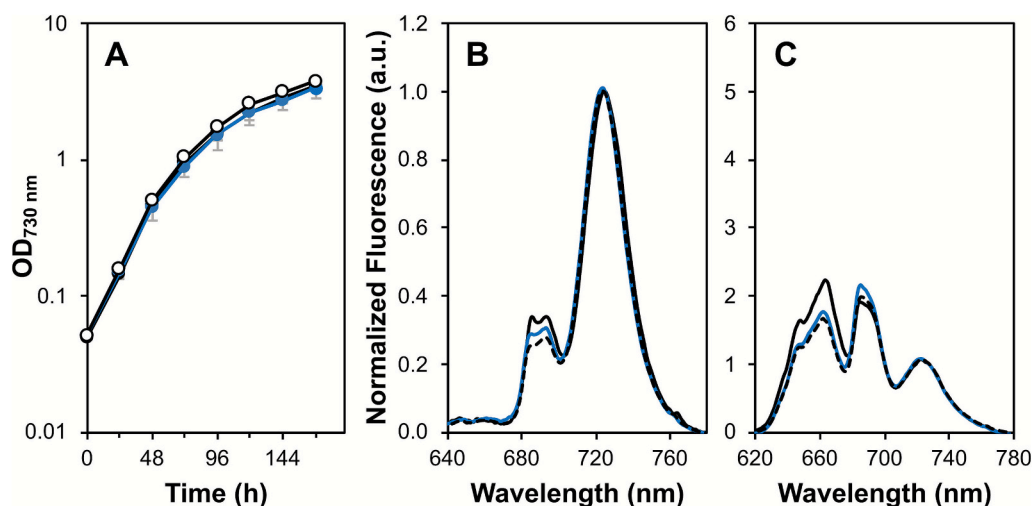


Fig. 2. Photoautotrophic growth and low-temperature (77 K) fluorescence emission spectra. (A) Photoautotrophic growth measured by turbidity at 730 nm. Control (black filled circles), R265A (blue filled circles) and R265D (black empty circles). Data are the average of three independent experiments and the standard error is shown. (B) Low-temperature emission spectra from cells excited at 440 nm. Control (black line), R265A (blue line) and R265D (black dashed line). (C) Low-temperature emission spectra from cells excited at 580 nm. Strains are the same as panel B. The low-temperature emission spectra are the average of three independent experiments and normalized to the PS I peak at 725 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

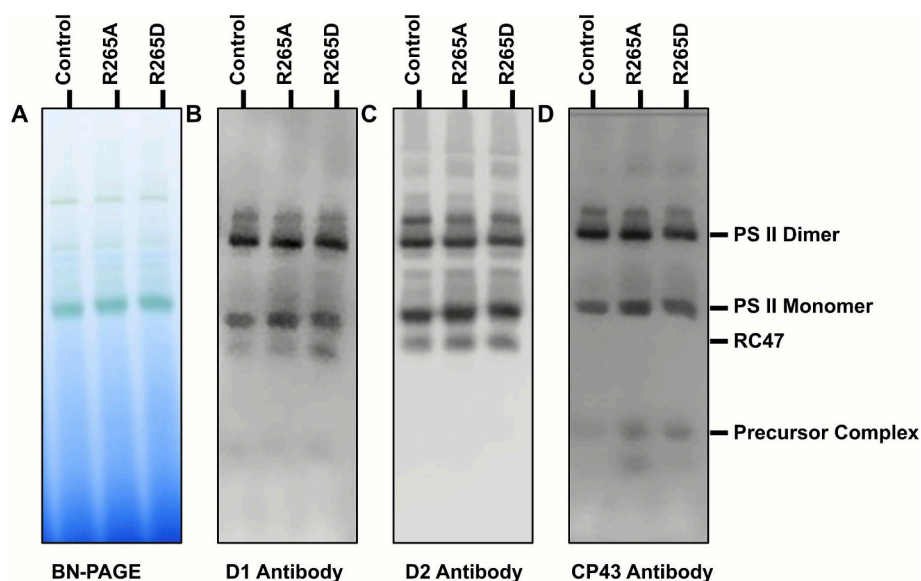


Fig. 3. Blue-native (BN) PAGE followed by western blot analysis. (A) BN-PAGE gel. (B) Western blot with antibody against D1. (C) Western blot with antibody against D2. (D) Western blot with antibody against CP43. The strains are labeled above their corresponding lane. RC47 is an assembly intermediate of PS II that lacks bound CP43. The experiment was repeated twice with similar results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Steady-state PS II activity is impaired in the R265A and R265D mutants

Variable chlorophyll *a* fluorescence induction assays were performed to assess electron transfer efficiency in the Arg-265 mutant strains. In the absence of DCMU, the control strain displayed a typical OJIP fluorescence induction curve, where the initial rise from F_0 (or O) to the inflection at J is due to the photochemical reduction of Q_A , and this is followed by a transition to a second inflection, I, and a peak at F_m (or P) as the downstream electron acceptors become reduced (Fig. 4A). Subsequently, the fluorescence declined due to the onset of the Calvin-Benson-Bassham cycle and various other quenching mechanisms [48]. Upon DCMU addition, chlorophyll *a* fluorescence rapidly reached F_m ,

reflecting inhibition of forward electron transfer from Q_A^- (Fig. 4B). The variable fluorescence yield in the presence of DCMU typically indicates the relative abundance of active PS II centers [43].

Both the R265A and R265D mutants displayed a significantly larger O–J rise than the control strain. Given that the actinic light intensity was kept constant, the larger O–J rise in the mutants suggests slower electron transfer from Q_A^- to Q_B (or Q_B^-). PS II activity in the D2-Arg265 mutants was therefore further examined using oxygen evolution assays performed in the presence of the PS II-specific electron acceptor DMBQ or with sodium bicarbonate, which supports whole chain electron transfer (Fig. 4C and D). In the presence of DMBQ, the R265A strain exhibited a reduction in oxygen evolution to 28 % of the control rate, while the R265D strain showed an even lower rate at 25 % (Fig. 4C and Table S2).

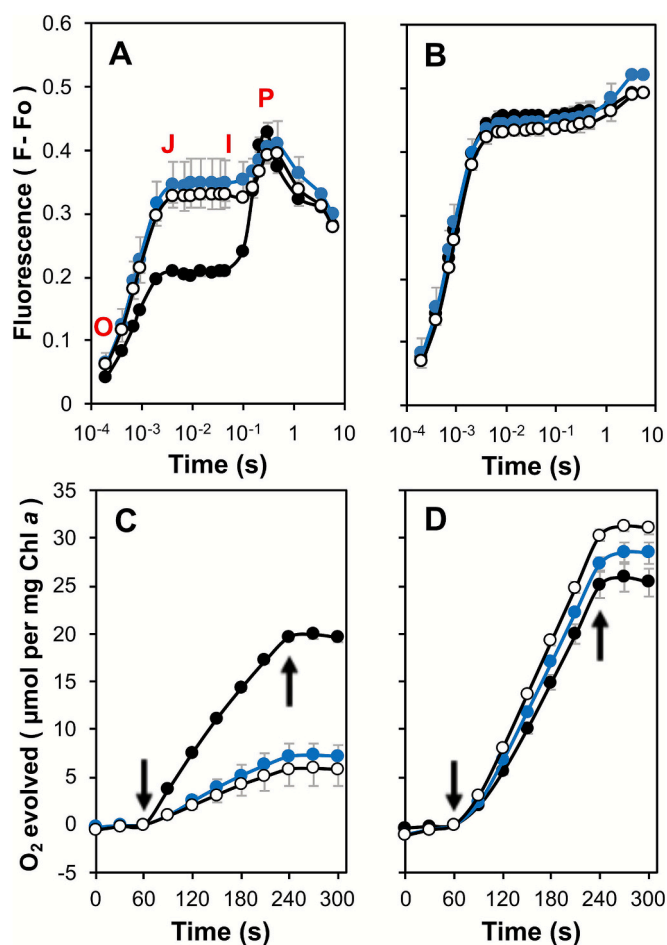


Fig. 4. Photosystem II activity in the strains with mutations at Arg265. (A) Variable chlorophyll *a* fluorescence in the absence of DCMU. Control (black filled circles), R265A (blue filled circles) and R265D (black empty circles). O is the origin, J and I inflections and P the peak of the observed fluorescence. F is the measured fluorescence at each time point and F₀ is the initial fluorescence in dark-adapted cells. (B) Variable chlorophyll *a* fluorescence in the presence of DCMU. Strains are the same as shown in panel A. (C) Oxygen evolution supported by 200 μM DMBQ and 1 mM K₃Fe(CN)₆. Arrows indicate light on and off. Control (black filled circles), R265A (blue filled circles) and R265D (black empty circles). (D) Oxygen evolution supported by 15 mM bicarbonate. Arrows indicate light on and off. Strains are the same as shown in panel C. Error bars represent the standard error from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

However, in the presence of bicarbonate, the oxygen evolution rate of the R265A strain increased to 103 % of the control, and the R265D strain exceeded the control by 16 % (Fig. 4D and Table S2). The elevated rate of whole chain electron transfer in the mutants may reflect an increased fraction of oxidized PQ to support linear electron flow because of reduced PS I cyclic electron transfer in these strains. Overall, these results suggest that D2-Arg265 mutations impair electron transfer between Q_A and Q_B and may potentially alter DMBQ binding in the mutants.

3.3. The R265A and R265D mutants exhibit enhanced sensitivity to high light

To investigate the stability of PS II in the R265A and R265D mutants, their sensitivity to high light was assessed to evaluate their susceptibility to photoinhibition and their capacity for recovery. High-light treatment at 2000 μmol photons m⁻² s⁻¹ was applied for 45 min, followed by

recovery under low light at 30 μmol photons m⁻² s⁻¹.

PS II-specific oxygen evolution was measured in the presence of DMBQ. Both mutants exhibited increased susceptibility to photodamage compared to the control (Fig. 5A). In high light, the rate of oxygen evolution in the R265A strain dropped to 8 % of its initial value, while the R265D mutant declined only to 20 %. Upon transfer to low light, a partial recovery was observed, with oxygen evolution in R265A cells reaching 53 % and R265D cells recovering to 60 % relative to their initial rates. In comparison, the control only dropped to 76 % of its initial rate under high light and exhibited a recovery to 137 % when returned to low-light conditions.

Whole chain electron transport, supported by bicarbonate, remained stable in high-light treated cells in contrast to the observed impairment of oxygen evolution supported by DMBQ (Fig. 5B). Hence, the native quinone was better able to operate in the photodamaged cells than DMBQ and suggests that sufficient cells remained active in both R265A and R265D mutants that, under the saturating light utilized in the assay, the rate-limiting step (i.e., PQH₂ oxidation by the cytochrome *b₆f* complex) remained similar in both mutants and control cells.

3.4. Decay of variable chlorophyll *a* fluorescence and thermoluminescence suggest the presence of an altered Q_B-binding site in the R265A and R265D strains

Given the slowing of electron transfer from Q_A⁻ indicated by variable fluorescence induction measurements, and the apparent altered electron acceptor side of PS II in response to high light impairing DMBQ-supported oxygen evolution, electron transfer from Q_A to Q_B was probed further, using the decay of chlorophyll *a* fluorescence following a single actinic flash. In the absence of DCMU, the fluorescence decay kinetics can be divided into three distinct phases [43]. The fast phase, occurring on the microsecond (μs) timescale, reflects the oxidation of Q_A⁻ by bound Q_B or Q_B⁻. This phase is followed by an intermediate phase in the millisecond (ms) range, which arises from the binding of Q_B to an unoccupied site and the subsequent oxidation of Q_A⁻. The final stage, or the slow phase, takes place on the seconds (s) timescale and corresponds to the back-reaction between Q_A⁻ and the S₂ state of the OEC. Fluorescence decay measurements conducted on the control strain exhibited a fast phase with a half-time (t_{1/2}) of 289 μs and an amplitude of 67 %, an

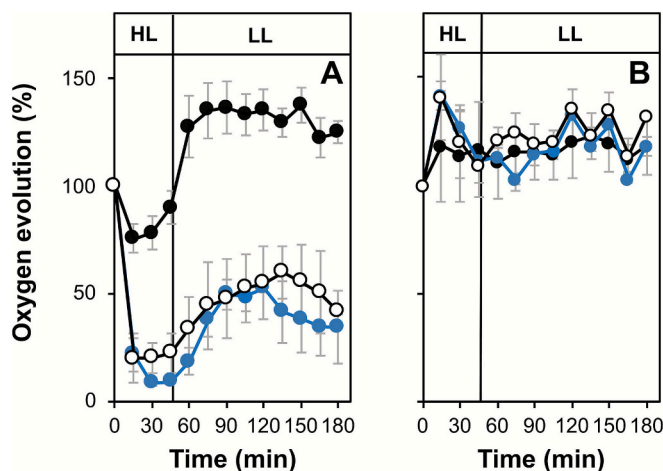


Fig. 5. Sensitivity of oxygen evolution to high light (or photodamage) in control and mutant cells. (A) Oxygen evolution measured in the presence of DMBQ and K₃Fe(CN)₆: Control (black filled circles), R265A (blue filled circles) and R265D (black empty circles). (B) Oxygen evolution in the presence of HCO₃⁻: symbols as in panel A. In both panels, high light (HL) was 2000 μmol photons m⁻² s⁻¹ and low light (LL) 30 μmol photons m⁻² s⁻¹. Error bars represent the standard error from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intermediate phase with a $t_{1/2}$ of 3 ms and an amplitude of 25 %, and a slow phase with a $t_{1/2}$ of 10 s and an amplitude of 8 % (Fig. 6A and B and Table 1).

The decay of fluorescence in the R265A and R265D mutants, however, was slowed in comparison to the control (Fig. 6A and B and Table 1). The R265A mutant exhibited a 2.7-fold increase in the half-time of the fast phase ($t_{1/2}$ 782 μ s), a 2.6-fold increase in the intermediate phase ($t_{1/2}$ 7.7 ms), and a 3.5-fold decrease in the slow phase ($t_{1/2}$ 2.8 s) relative to the control strain. Additionally, a substantial increase in the amplitude was observed in the intermediate (34 %) and slow (16 %) phases when compared to the control. Similarly, the R265D mutant showed a 2.8-fold increase in the fast phase ($t_{1/2}$ 812 μ s), a 4.6-fold increase in the intermediate phase ($t_{1/2}$ 13.7 ms), and a 2.3-fold decrease in the slow phase ($t_{1/2}$ 4.3 s), but it only exhibited an increase in the amplitude at the slow phase (14 %). These results suggest that Q_A^- to Q_B electron transfer rates as well as Q_B binding were inhibited, leading to an equilibrium shift favouring the $Q_A^-Q_B$ state in the R265A and R265D mutants. In the presence of DCMU, both R265A and R265D mutants exhibited kinetics similar to the control indicating the back reaction between Q_A^- and S2 was not affected (Fig. 6C and D).

Thermoluminescence assays were conducted to further examine the impact on the acceptor side of PS II resulting from the introduction of Ala and Asp substitutions at D2-Arg265. Exposure of cells to a single actinic light flash produces three distinct TL bands (denoted B, C and Q), each associated with specific charge recombination events. The B band, detected at 29 °C in control cells, corresponds to $S_2Q_B^-/S_3Q_B^-$ recombination. The C band, appearing at 53 °C in the control, is attributed to charge recombination between Y_D^+ and Q_A^- , where Y_D represents D2-Tyr160 [49,50]; while the Q band, observed at 20 °C in the control, is indicative of $S_2Q_A^-$ recombination and is measured in the presence of DCMU [44,51,52].

Both the R265A (26 °C) and R265D (26 °C) mutants exhibited a shift in the B-band to a lower temperature compared to the control (Fig. 6E and Table 2). This suggests a more negative redox midpoint potential for the Q_B/Q_B^- couple, leading to a destabilized Q_B^- population. In the presence of DCMU, the Q-band in the R265A (23 °C) and R265D (22 °C) strains appeared at a slightly higher temperature relative to the control strain (Fig. 6F and Table 2), consistent with the fluorescence decay measurements in the presence of DCMU (Fig. 6D). However, a slight shift in the C-band in the absence of DCMU was observed for both the R265A (51 °C) and R265D (51 °C) mutants (Fig. S2 and Table 2).

Interestingly, despite chlorophyll *a* fluorescence induction assays indicating similar PS II levels in control cells and in the R265A and R265D strains (Fig. 4B), a lower TL intensity was observed in both the mutants. This suggests that in both mutants, non-radiative charge recombination pathways play a greater role than radiative pathways when compared to the situation in control cells, further supporting the hypothesis of a perturbed acceptor-side environment (Brinkert et al., 2016).

3.5. R265A and R265D cells exhibit enhanced sensitivity to formate and impaired Q_B exchange with the PQ pool that are partially reversed by the addition of bicarbonate

The D2-Arg265 residue forms a bond with D2-His268, a key histidine involved in the coordination of the NHI between Q_A to Q_B (Fig. 1). The additional presence of bicarbonate as a bidentate ligand to the NHI is essential for efficient electron transfer from Q_A to Q_B and its absence significantly impairs this process, although normal function can be restored upon the reintroduction of bicarbonate [27,28,53]. Furthermore, sodium formate (HCO_2^-) has been shown to compete with bicarbonate, effectively displacing bicarbonate and thereby inhibiting the electron transfer from the reduced Q_A to Q_B [54–58]. Therefore, the interaction between D2-Arg265 and D2-His268 likely plays a crucial role in stabilizing the bicarbonate-binding environment, ensuring efficient electron transfer between Q_A and Q_B .

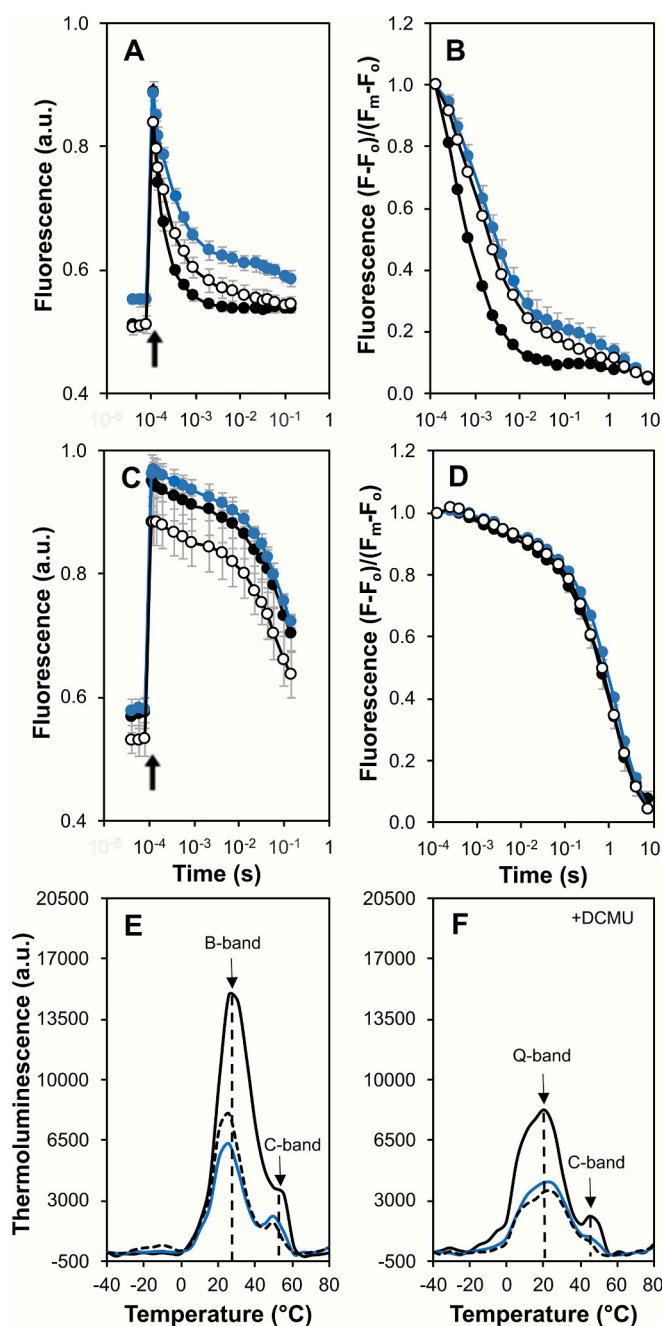


Fig. 6. Chlorophyll *a* fluorescence decay after a single turnover actinic flash and thermoluminescence emission in the strains with mutations at D2-Arg265. (A) Fluorescence decay after three measurements determining the initial (or F_0) fluorescence. Control (black filled circles), R265A (blue filled circles) and R265D (black empty circles). (B) Fluorescence decays from panel A normalized to the maximum fluorescence (F_m) where F is the fluorescence at each time point after the actinic flash. (C) Fluorescence decay after three measurements determining F_0 in the presence of DCMU. Strains and symbols are the same as panel A. (D) Fluorescence decays from panel C normalized to F_m . In panels A–D the data are the average of three independent experiments. Error bars represent the standard error. (E) Thermoluminescence emission in the absence of DCMU. Control (black line), R265A (blue line) and R265D (black dashed lines). (F) Thermoluminescence emission in the presence of DCMU. Strains are the same as panel E. In panels E and F, the data are the average of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1Kinetic analysis of chlorophyll *a* fluorescence decay following a single saturating actinic flash in strains with mutations at Arg265.

Strain	Treatment	Fast Phase		Intermediate Phase		Slow Phase	
		Rate $t_{1/2}$ (μ s)	Amplitude (%)	Rate $t_{1/2}$ (ms)	Amplitude (%)	Rate $t_{1/2}$ (s)	Amplitude (%)
Control	No treatment	289 $\pm$ 28	67.3 $\pm$ 1.7	3.0 $\pm$ 0.3	25.0 $\pm$ 1.0	9.8 $\pm$ 1.6	7.7 $\pm$ 0.8
	DCMU			2.0 $\pm$ 0.1	9.8 $\pm$ 0.1	0.7 $\pm$ 0.1	90.2 $\pm$ 0.1
R265A	No treatment	782 $\pm$ 83	49.7 $\pm$ 1.5	7.7 $\pm$ 1.0	34.2 $\pm$ 3.4	2.8 $\pm$ 0.4	16.2 $\pm$ 1.9
	DCMU			2.8 $\pm$ 0.2	8.3 $\pm$ 0.3	1.0 $\pm$ 0.1	91.8 $\pm$ 0.3
R265D	No treatment	812 $\pm$ 37	61.3 $\pm$ 0.7	13.7 $\pm$ 1.1	24.3 $\pm$ 0.6	4.3 $\pm$ 0.5	14.4 $\pm$ 0.8
	DCMU			2.4 $\pm$ 0.3	9.7 $\pm$ 0.2	0.7 $\pm$ 0.1	90.3 $\pm$ 0.3

The experiment was repeated three times, and the standard error is shown.

Table 2Thermoluminescence temperature maxima for the Q-band, B-band and C-band in *Synechocystis* sp. PCC 6803 mutants with substitutions at Arg265 of the D2 protein.

Strain	Q band ($^{\circ}$ C)	B band ($^{\circ}$ C)	C band ($^{\circ}$ C)
Control	20.4 $\pm$ 0.2	29.0 $\pm$ 1.0	53.4 $\pm$ 0.6
R265A	22.8 $\pm$ 0.5	25.8 $\pm$ 0.2	51.4 $\pm$ 0.7
R265D	22.3 $\pm$ 1.5	25.5 $\pm$ 1.0	50.7 $\pm$ 0.5

The experiment was repeated three times, and the standard error is shown.

To assess the impact of the D2-Arg265 substitutions on bicarbonate binding and electron transfer, fluorescence decay kinetics were measured following the separate and combined addition of bicarbonate and formate. In the presence of bicarbonate, the chlorophyll *a* fluorescence decay kinetics in the control strain remained similar to that under untreated conditions (Fig. 7A and Table S3). The R265A and R265D mutants, however, exhibited an accelerated fast phase (450 μ s [52 %] and 428 μ s [52 %], respectively) and intermediate phase (4.4 ms [37 %] and 4.3 ms [38 %], respectively) and a delayed slow phase (6.5 s [12 %] and 8.4 s [10 %], respectively) relative to no treatment (Fig. 7B and C and Table S3). Although these values reflect improved kinetics, they were not fully restored to the control levels. This suggests that while bicarbonate improves electron transfer efficiency, it does not completely compensate for the perturbations caused by the mutations.

The introduction of formate to the R265A and R265D mutants resulted in a pronounced delay in the fast (1097 μ s [39 %] and 1092 μ s [31 %], respectively) and intermediate fluorescence decay phases (13.0 ms [31 %] and 19.0 ms [41 %], respectively), while the slow phase was accelerated (1.1 s [30 %] and 0.8 s [28 %], respectively), accompanied by a greater contribution of the intermediate and slow phases. This observation suggests that formate effectively displaced bicarbonate from its binding site in these mutants, thereby disrupting Q_A^- to Q_B electron transfer and shifting the equilibrium for the sharing of an electron between Q_A and Q_B towards Q_A^- . Reintroduction of bicarbonate successfully restored the decay kinetics in both mutants, highlighting the importance of D2-Arg265 in maintaining an optimal bicarbonate binding environment and influencing the susceptibility towards formate addition.

Furthermore, when the mutants were exposed to multiple actinic flashes, a significant further delay in chlorophyll *a* fluorescence decay was observed under untreated conditions and in the presence of formate, suggesting that the formation of Q_BH_2 and its exchange with PQ were compromised (Fig. 7E and F). This impairment was not observed when the sample was treated with bicarbonate alone or in combination with formate, further supporting the notion of a perturbed bicarbonate-binding environment in the mutants.

Moreover, the addition of bicarbonate partially alleviated the O–J rise in the R265A and R265D mutants, though it did not fully restore the kinetics to control levels during the variable chlorophyll fluorescence induction assay (Fig. S3). In the presence of formate, the Q_A^- to Q_B electron transfer appeared to slow down further, as indicated by the elevated J level coinciding with Fmax (or the P level). However, the

addition of bicarbonate was effective in reversing the inhibitory effects of formate (Fig. S3).

To further investigate the role of bicarbonate and formate in electron transfer, oxygen evolution assays were conducted using DMBQ as an artificial electron acceptor. In the presence of both DMBQ and bicarbonate, oxygen evolution rates in the R265A and R265D strains were increased relative to when DMBQ was present alone, suggesting that bicarbonate enhances PS II electron transfer efficiency (cf. Fig. 4C and Fig. 7G).

Conversely, in the presence of formate, a significant reduction in oxygen evolution was observed in both the R265A and R265D strains (Fig. 7H). This effect was more pronounced than when only DMBQ was present, further indicating that formate inhibits forward electron transfer by displacing bicarbonate. However, when bicarbonate was reintroduced, the oxygen evolution rates were restored in all the strains, consistent with bicarbonate playing a role in mitigating the impaired electron transfer in the mutants (Fig. 7I).

Overall, these findings suggest that D2-Arg265 mutations weaken bicarbonate binding, making it more susceptible to displacement by formate. This results in impaired electron transfer from the reduced Q_A to Q_B , slower PQ exchange, and reduced oxygen evolution rates, all of which are partially alleviated by bicarbonate replenishment.

3.6. Forward electron transfer from Q_A^- in the R265A and R265D mutants is inhibited in the presence of DMBQ

The introduction of DMBQ as an artificial electron acceptor resulted in distinct kinetic differences between the control strain and the R265A and R265D mutants during the variable chlorophyll *a* fluorescence induction assay (Fig. 8A). In the control strain, the presence of DMBQ induced a prolonged J–I–P rise, which is attributed to the delayed reduction of the PQ pool and the subsequent accumulation of Q_A^- . This phenomenon is characteristic of an artificial electron acceptor like DMBQ, which interacts with the Q_B -binding site differently than the native PQ, due, for example, to its lack of a long isoprenoid tail.

In contrast, the R265A and R265D mutants exhibited a significantly larger O–J rise when exposed to DMBQ, with induction kinetics resembling those observed in the presence of DCMU. This similarity suggests that Q_A^- oxidation is severely inhibited in the mutants, likely due to alterations in the Q_B -binding site. Enhanced accessibility or stronger binding of DMBQ at the Q_B site could potentially slow down Q_A^- oxidation and disrupt normal electron transfer. In addition, the likely presence of a more oxidized PQ pool in the two mutants may place the cells in State I (preferential energy transfer from the phycobilisome to PS II) with a resulting increase in fluorescence yield [59].

Further evidence supporting the above interpretation comes from the observation that the R265A and R265D mutants display a 5.2-fold ($t_{1/2}$, 4077 μ s) and 3.8-fold ($t_{1/2}$, 3061 μ s) increase in the apparent half-time of their fast phase, respectively, compared to untreated conditions during chlorophyll *a* fluorescence decay assays (Fig. 8B and Table S3). Additionally, the mutants exhibited a significantly larger contribution of their slow phase, with R265A at 45 % and R265D at 41 %. The increased contribution of the slow phase further implies that the Q_B -binding area is

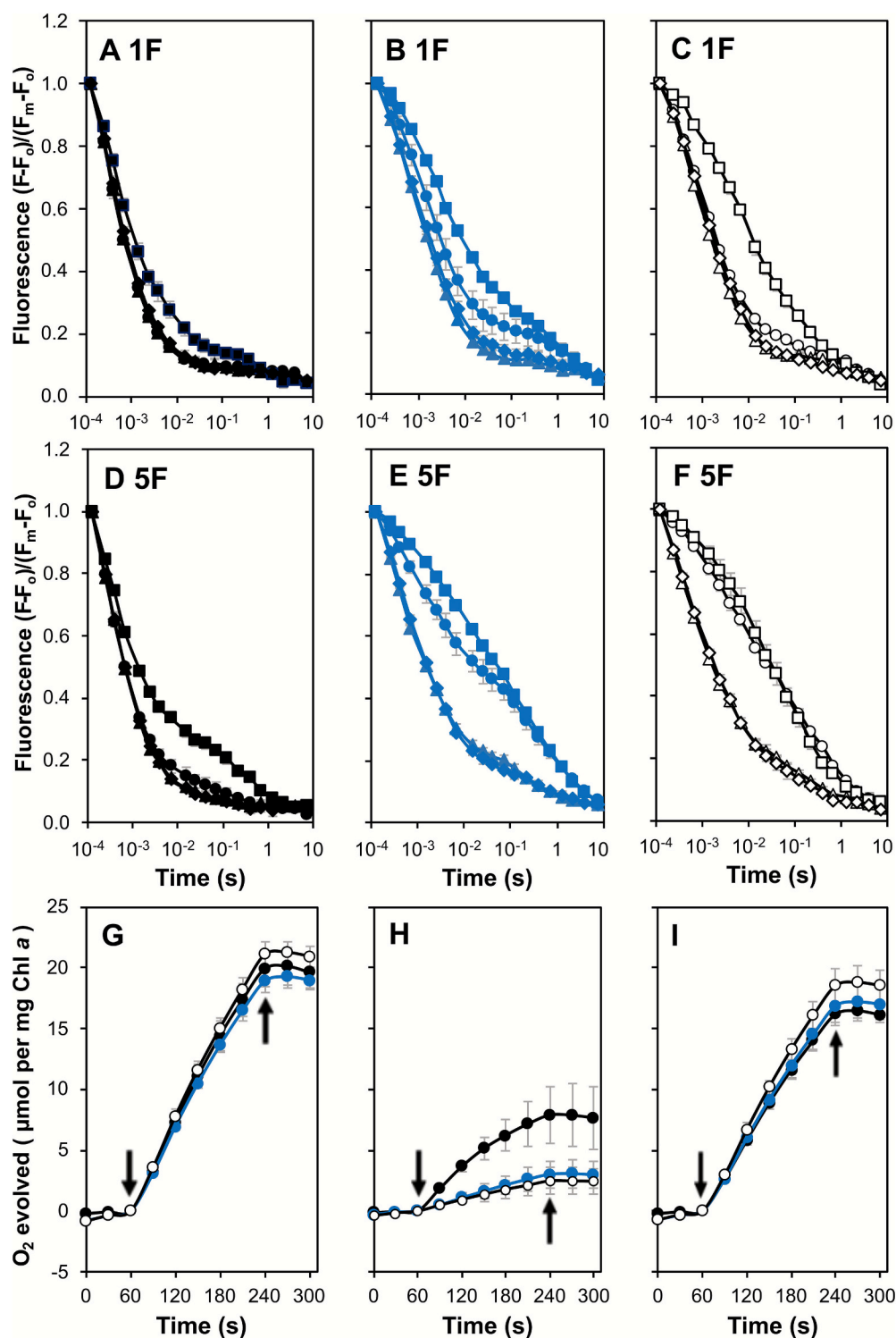


Fig. 7. Chlorophyll *a* fluorescence decay and oxygen evolution in the presence of bicarbonate, formate or bicarbonate and formate in strains with mutations at D2-Arg265. (A) Fluorescence decay after one actinic flash in control cells. No treatment (black filled circles), 15 mM bicarbonate (black filled triangles), 25 mM formate (black filled squares) and 15 mM bicarbonate and 25 mM formate (black filled diamonds). (B) Fluorescence decay after one actinic flash in R265A cells. No treatment (blue filled circles), 15 mM bicarbonate (blue filled triangles), 25 mM formate (blue filled squares), 15 mM bicarbonate and 25 mM formate (blue filled diamonds). (C) Fluorescence decay after one actinic flash in R265D cells. No treatment (black empty circles), 15 mM bicarbonate (black empty triangles), 25 mM formate (black empty squares), 15 mM bicarbonate and 25 mM formate (black empty diamonds). (D) Fluorescence decay after five actinic flashes given at 5 Hz in control cells. Symbols as in panel A. (E) Fluorescence decay after five actinic flashes given at 5 Hz in R265A cells. Symbols as in panel B. (F) Fluorescence decay after five actinic flashes given at 5 Hz in R265D cells. Symbols as in panel C. F, F_0 and F_m in panels A–F are defined in the legend to Fig. 6. (G) Oxygen evolution in the presence of 15 mM bicarbonate and 200 μM DMBQ. Control (black filled circles), R265A (blue filled circles) and R265D (black empty circles). (H) Oxygen evolution in the presence of 25 mM formate and 200 μM DMBQ. Strain identification as in panel G. (I) Oxygen evolution in the presence of 15 mM bicarbonate, 25 mM formate and 200 μM DMBQ. Strain identification as in panel G. In all assays of oxygen evolution, 1 mM $K_3Fe(CN)_6$ was added together with DMBQ. Arrows indicate actinic light on and

off. All data in this figure are the average of three independent experiments. Error bars represent the standard error. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

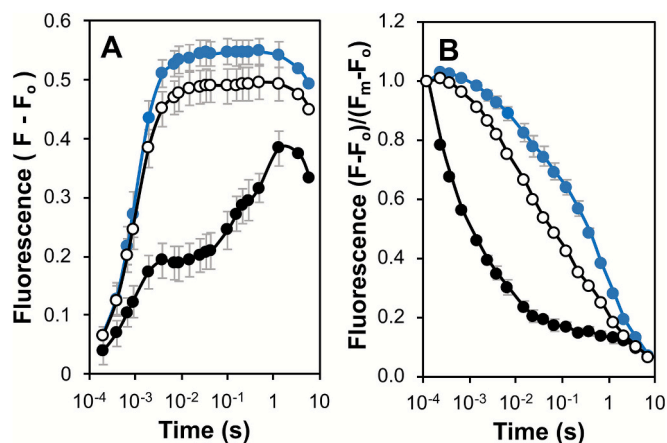


Fig. 8. Variable chlorophyll *a* fluorescence and decay in the presence of DMBQ in strains with mutations at D2-Arg265. (A) Variable chlorophyll *a* fluorescence in the presence of DMBQ. Control (black filled circles), R265A (blue filled circles) and R265D (black empty circles). *F* and *F*₀ are defined in the legend to Fig. 4. (B) Chlorophyll *a* fluorescence decay after a single saturating actinic flash in the presence of DMBQ. Control (black filled circles), R265A (blue filled circles) and R265D (black empty circles). *F*, *F*₀ and *F*_m are defined in the legend to Fig. 6. Error bars represent the standard error from three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

perturbed, allowing DMBQ to bind with greater affinity than in the control strain.

These findings are consistent with an inverse relationship between the binding strength of artificial electron acceptors and oxygen-evolving activity (e.g., Fig. 4C) [60]. The requirement for artificial acceptors to dissociate before the next electron acceptor can bind suggests that any perturbation of the Q_B-binding site altering DMBQ exchange or binding affinity could significantly inhibit Q_A oxidation. Moreover, the R265A and R265D mutants exhibited normal chlorophyll *a* fluorescence decay kinetics in the presence of DCMU (Fig. 6D), indicating that the observed impairment reflects inhibited forward electron flow in the presence of DMBQ because of an altered Q_B-binding environment in the mutants.

4. Discussion

4.1. The contribution of D2-Arg265 to the symmetry of the quinone-Fe-acceptor complex

The four His residues coordinating the NHI in PS II are provided by D1-His215 and D1-His272, and D2-His214 and D2-His268; and these are flanked by D1-Arg269 and D2-Arg265, respectively. The coordination sphere of the Fe is completed by bidentate ligation by the bicarbonate ion (Fig. 1). The D1-Arg269 residue has putative hydrogen bonds between its backbone carbonyl group and the N π nitrogen of the imidazole ring and the backbone amide of D1-His272. The N π nitrogen of D1-His272 also has a hydrogen bond with the backbone carbonyl from the adjacent D1-Ser268. This configuration is mirrored by the interactions of D2-Arg265 and D2-Lys264 with D2-His268 (Fig. 1). The substitution of D1-Arg269 with a Gly residue in *Chlamydomonas reinhardtii* resulted in the loss of the Mn₄CaO₅ cluster or OEC [31]. In addition, electron transfer from reduced Q_A to Q_B was impaired and herbicide-binding at the Q_B-binding site was destabilized [32]. Hence, the impact of mutating D1-Arg269 impacted both the donor and the acceptor sides of PS II. Given the importance of D1-Arg269 we have addressed the role of the corresponding D2-Arg265. To conduct this

study, we introduced an alanine substitution to investigate the importance of the D2-Arg265 side chain, and we also examined the importance of its positive charge by replacing D2-Arg265 with an aspartate.

4.2. The participation of D1-Ser268 and D2-Lys264 in the Q_A-Fe-Q_B complex

D1-Arg269 together with D1-Ser268 (in the vicinity of the Q_B-binding site) and D2-Arg265 together with D2-Lys264 (in the vicinity of the Q_A-binding site) form corresponding residue pairs stabilizing His ligation of the NHI (Fig. 1). As mentioned earlier, formate has been widely used to displace the bicarbonate ion from the NHI [54,56]. Due to the disruption arising from the introduction of Gly at D1-Arg269 the extent of bicarbonate-reversible formate inhibition in this mutant, relative to wild type, was unclear [32]. However, mutations at D1-Ser268 have been shown to increase the sensitivity to formate [58]. D1-Ser268 has also been implicated in an alternative route for the protonation of Q_B⁻ (H⁺) [58]. Typically, in the formation of Q_BH₂, the first proton is supplied via D1-His252 and D1-Ser264, while the second proton is provided by D1-His215 [9,61]; in this pathway, re-protonation of D1-His215 is accomplished by the hydrogen-bond network incorporating bicarbonate (Fig. 1). It has been suggested that when bicarbonate-binding is disrupted by the addition of formate, D1-Ser268 participates in an alternative pathway to deliver a proton to Q_B⁻ (H⁺) [58].

Mutations targeting D2-Lys264 in *Synechocystis* 6803 have been shown to impair electron transfer through the Q_A-Fe-Q_B complex and to perturb PS II assembly [34]. In addition to hydrogen bonding to the D2-His268 N π nitrogen, D2-Lys264 has hydrogen-bonding or salt-bridge interactions that may account for these observations. These may impact the accessibility of water to the NHI [62–64]. In addition, disruption of the hydrogen bond between D2-Lys264 and D1-Glu244 may contribute to destabilization of the bicarbonate-binding environment since mutations targeting D1-Glu244 have been shown to alter the Q_B-binding environment and to increase the apparent redox potential of the Q_A/Q_A⁻ couple [57]. Furthermore, the D2-K264E mutant, which exhibited impaired electron transfer between Q_A and Q_B, was insensitive to both bicarbonate and formate addition and the D1-E244A mutant likewise had impaired electron transfer between Q_A and Q_B and was also insensitive to both bicarbonate and formate addition [34,57].

The corresponding residue to D1-Glu244 in D2 is D2-Glu241. Prior to CP43 binding to the RC47 complex during PS II assembly, D2-Glu241 acts as a monodentate ligand to the NHI potentially contributing to the prevention of premature bicarbonate binding along with the displacement of the DE-loop of D1 that is aided by the presence of the Psb28 assembly factor [15,65] (Fig. S4). The “bicarbonate off” configuration of the quinone-Fe complex would likely favor photoprotective cyclic electron transfer around PS II, while the switch to bound bicarbonate and the accompanying occupancy of the Q_B-binding site would favor photoactivation of the OEC [15,29,65,66].

4.3. D1-Thr245 and D2-Thr243 interact with D2-Arg265 and D1-Arg269

A conserved motif associated with bicarbonate binding is present within the DE-loops of D1 (²⁴²EEETYNIVA²⁵²) and D2 (²⁴⁰AEE-TYSMTAN²⁵⁰) which positions D1-Tyr246 and D2-Tyr244 to stabilize the bicarbonate (Fig. 1) [4,57,67,68]. In addition, the residues within this motif participate in the hydrogen bond network including a preserved salt bridge (or hydrogen bond) between the hydroxyl of D2-Thr243 and the N ϵ nitrogen of D1-Arg269 and the salt bridge (or hydrogen bond) between D1-Thr245 and an N η nitrogen of D2-Arg265 (Fig. 1).

4.4. Phenotypic consequences of mutations targeting D2-Arg265

Mutations targeting D2-Arg265 had less impact on PS II assembly than mutations targeting D2-Lys264. Both R265A and R265D cells exhibited similar photoautotrophic growth and PS II assembly to control cells (Figs. 2 and 3), whereas the K264A and K265E mutants exhibited reduced assembly [34]. Mutations targeting D2-Arg265 and D2-Lys264 also differed in their response to high light. When supported by the PS II-specific electron acceptor DMBQ, oxygen evolution was rapidly inactivated in R265A and R265D cells and did not recover under low light (Fig. 5A). In contrast, oxygen evolution in K264E cells was similar to that in the control, and while the K264A strain did undergo photodamage during exposure to 2000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, these cells recovered under low light (30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

Whole chain electron transport supported by bicarbonate also produced different results between the strains. In particular, the K264E cells underwent photodamage and then were unable to recover, whereas both R265A and R265D mutants, as well as K264A cells, retained rates of oxygen evolution like control cells throughout the high-light treatment. This suggests the K265E mutant experienced irreversible inactivation and in fact K265E cells were found to be unresponsive to both formate and bicarbonate addition [34]. Nevertheless, although it appeared that the native quinone could support whole chain rates of electron transport in R265A and R265D cells at a comparable rate to that of control cells, both chlorophyll *a* fluorescence induction and decay measurements indicated that the binding of DMBQ was altered and the electron transfer from Q_A^- was retarded in both mutants (Fig. 8). Thermoluminescence measurements also indicated a modified Q_B -binding site with the apparent midpoint potential of the $\text{Q}_\text{B}/\text{Q}_\text{B}^-$ couple becoming more negative (Fig. 6E and Table 2).

The R265A and R265D mutants also exhibited bicarbonate-reversible impaired electron transfer from Q_A^- to Q_B as indicated by both variable chlorophyll *a* induction measurements and the decay of variable fluorescence after a single actinic flash (Fig. 7A-C, Fig. S3, and Table S3). Moreover, while the decay of chlorophyll *a* fluorescence was more susceptible to formate in the mutants than in control cells, after multiple turnovers of PS II, the decays were not only more severely impaired, but additionally, formate had little additive effect. This suggests that the exchange of $\text{Q}_\text{B}\text{H}_2$ with the PQ pool was impaired in both R265A cells and R265D cells (Fig. 7D-F). A similar impairment of quinol exchange was apparent when mutations were introduced at D2-Lys264 [34]. In comparison, while the D1-S268A and D1-S268T mutants were more susceptible to formate than the control cells, their susceptibility was less than that observed in the R265A and R265D mutants. Direct comparison with the corresponding D1-Arg269 position was not possible since the D1-R269G cells also failed to assemble any OEC and no donor side effect was observed in any of the mutants with substitutions at D2-Lys264, D2-Arg265 and D1-Ser268. It should be noted, however, that the D1-R269G strain was constructed in *C. reinhardtii*, and therefore a species-specific effect cannot be ruled out [31,32].

4.5. The bicarbonate-specific effect on PS II assembly and quinol formation

Directed mutagenesis of the bicarbonate-binding motif, as well as the D2-Arg265/D2-Lys264 and D1-Ser268/D1-Arg269 pairs, has demonstrated a key role for bicarbonate and the bicarbonate-binding environment in quinol reduction [34,57,58,67–70]. In contrast, the DE-loops, and the apparent stabilization of the His ligands to the NHI by Arg residues, are not observed in the quinone-Fe acceptor complex of anoxygenic photosynthetic bacteria (Fig. S5). This raises the question of what is the advantage of incorporation of the bicarbonate ion into the quinone-iron-complex that is seen in oxygenic photosynthesis?

It has been shown that the accumulation of reduced Q_A^- is accompanied by bicarbonate release from the NHI and this increases the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ redox potential to favor direct recombination with P680^+ .

Under environmental conditions where Q_A^- might accumulate (e.g., a combination of high light and low temperature) this would provide photoprotection by decreasing backreactions that otherwise would lead to $^1\text{O}_2$ formation via ^3Chl formed through the back reaction pathway [29]. Similar photoprotection of PS II, while the Q_B -binding site is empty during biogenesis, might also occur prior to the binding of bicarbonate and Q_B that precedes the onset of photoactivation (see above). It has been suggested that the mechanism underlying the shift in the $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ redox potential involves protonation of Q_A^- via D2-His214 following bicarbonate release and that the release of bicarbonate proceeds via an intermediate step involving bicarbonate as a monodentate ligand to the NHI [30]. In the R265A and R265D cells, our TL measurements were consistent with an increased $\text{Q}_\text{A}/\text{Q}_\text{A}^-$ redox potential; however, both strains exhibited an increased sensitivity to high light, suggesting the mutants had lost their ability to undergo this photoprotective process. A structure for a putative dimeric Psb27:PS II intermediate complex lacking the PsbO, PsbU and PsbV extrinsic proteins was found to have bicarbonate as a monodentate ligand and both D2-H268 and D1-His272 likely no longer coordinated the NHI in this complex [71] (Fig. S6). Further studies are required, however, to establish if the Psb27:PS II complex is typical of the PS II complexes involved in the lifecycle of PS II [21].

5. Conclusion

While mutations targeting D2-Arg265 had a minor impact on PS II assembly, D2-Arg265 was found to be instrumental in stabilizing bicarbonate binding, maintaining forward electron transfer, and influencing PQH_2 exchange between the Q_B -binding site and the PQ pool. The introduced mutations in the R265A and R265D strains resulted in inhibited Q_A^- to Q_B electron transfer, increased sensitivity to high light, and an altered Q_B -binding site; and this may arise, in part, through destabilization of the coordination of the NHI by D2-His268 and bicarbonate. These findings underscore the importance of the hydrogen bond network associated with the electron acceptor side of PS II and support the notion that the architecture of the protein environment associated with the binding of Q_A and Q_B in oxygenic photosynthesis plays a role in photoprotection, and moderating photoactivation of the OEC, in addition to supporting proton-coupled electron transfer and quinol exchange with the PQ pool [15,29,65,66].

CRedit authorship contribution statement

Victor Zhong: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Imre Vass:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Julian J. Eaton-Rye:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbabo.2025.149578>.

Data availability

Data will be made available on request.

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