Mutations of cytochrome b_{559} and PsbJ on and near the Q_C site in photosystem II influence the regulation of short-term light response and photosynthetic growth of the cyanobacterium *Synechocystis* sp. PCC 6803

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Abbreviations

AL, actinic light; APC, allophycocyanin; Chl, chlorophyll; Cyt, cytochrome; E_m, erythromycin; F_m, the maximal fluorescence yield; F_{mdark}, the maximal fluorescence yield in the dark; FRP, fluorescence recovery protein; F_o, the dark fluorescence yield; $F_v/F_{m,}$ the maximal PSII quantum yield [with $F_v = (F_m-F_o)$]; GFP, green fluorescent protein; M, phycobilisome-free membrane; MP, phycobilisome-associated membrane; NPQ, nonphotochemical fluorescence quenching; OCP, orange carotenoid proteins; PB, phycobilisome; PQ, plastoquinone; PSII, photosystem II; Q_A, the primary quinone electron acceptor in PSII; Q_B, the secondary quinone electron acceptor in PSII; Q_C, the third plastoquinone-binding site in PSII; Sp, streptomycin; TL, thermoluminescence; UPLC-APCI-QTOFMS; ultra performance liquid chromatography-atmospheric pressure chemical ionization-quadrupole time-of-flight mass spectrometry; WT, control *Synechocystis* strain constructed the same as site-directed mutants but with no mutation

ABSTRACT

The characteristic features of two types of short-term light adaptations of the photosynthetic apparatus of the cyanobacterium *Synechocystis* sp. PCC 6803, state transition and blue-green light-induced fluorescence quenching, were compared in wild-type and cytochrome (Cyt) b_{559} and PsbJ mutant cells with mutations on and near the Q_C site in photosystem II (PSII). All of the mutant cells grew photoautotrophically and assembled stable PSII. Thermoluminescence emission experiments showed a decrease in the stability of the $S_3Q_B^-/S_2Q_B^-$ charge pairs in the A16FJ, S28Aβ and V32Fβ mutant cells. When dark-adapted wild-type and mutant cells were illuminated by medium-intensity blue light, the increase in the PSII fluorescence yield (indicating a transition to state 1) was more prominent in mutant than wild-type cells. Strong blue-light conditions induced a quenching of fluorescence corresponding to non-photochemical fluorescence quenching (NPQ). The extension of NPQ was significantly decreased in the mutants and the kinetics appeared to be affected. When similar measures were repeated on an orange carotenoid protein (OCP)-deficient background, little or no quenching was observed, which confirms that the decrease in fluorescence under strong blue light corresponded to the OCP-dependent NPQ. Immunoblot results showed that the attenuated effect of blue-light-induced NPQ in mutant cells was not due to lack of OCP. Photosynthetic growth and biomass production were greater for A16FJ, S28Aβ and V32Fβ mutant cells than wild-type cells under normal growth conditions. Our results suggest that mutations of Cyt b_{559} and PsbJ on and near the Q_C site of PSII may modulate the short-term light response in cyanobacteria.

Introduction

Cyanobacteria have developed short-term light responses that do not involve changes in gene expression. The two principal ones are state transitions and orange carotenoid protein (OCP)-related non-photochemical fluorescence quenching (NPQ). The former denotes the ability to distribute excitation energy between photosystem I and photosystem II (PSII) as a function of the redox state of the plastoquinone (PQ) pool.[1-5] When cyanobacteria are illuminated with orange light, principally absorbed by phycobilisomes (PBs), the PQ pool becomes more reduced, which induces a transition to state 2 with decreased PSII fluorescence yield. In contrast, blue or red light, principally absorbed by chlorophyll (Chl), induces a transition to state 1 with increased PSII fluorescence yield. In darkness, *Synechocystis* sp. PCC 6803 cells are in state 2, because the PQ pool is reduced via respiration. Thus, illumination of dark-adapted cells with low intensities of blue or red light provokes a transition to state 1.

The OCP-related NPQ of cyanobacteria is a photoprotective mechanism which reduces the energy arriving to the reaction centers under strong light conditions.[6-8] Under strong white or blue light, the OCP undergoes photo-conversion into the active red form, which interacts with the allophycocyanin (APC) core of the PB to induce the NPQ effect, dissipating excess excitation energy on the PB as heat and thereby protecting PSII reaction centers against photodamage.[6-18] During this process, the OCP acts as the light sensor, signal propagator and energy quencher.[16] With decreased blue or white light intensity, the binding between the red form of OCP and APC core of the PB is released with the help of fluorescence recovery protein (FRP). FRP interacts with the C-terminal domain of the red form of OCP and accelerates its conversion to the orange form and detachment from the PB to stop the NPQ process.[10] In contrast to knowledge of photoprotective mechanisms in higher plants and algae,

evidence is lacking for a feedback regulation of PSII in OCP-mediated photoprotection in cyanobacteria.[19]

Cyt b_{559} is one of the essential components of PSII reaction center, but its function, although widely investigated, still remains unclear.[20, 21] Previous studies have proposed that Cyt b_{559} may participate in secondary electron transfer pathways protecting PSII against oxidative damage.[20-22] In these proposals, Cyt b_{559} would donate electrons, via a β-carotene molecule (Car_{D2}), to reduce light induced Chl radicals and prevent the formation of reactive oxygen species in PSII under donor-side photoinhibitory conditions (*e.g.*, the Mn_4O_5Ca cluster is impaired or under assembly).[22-24] On the other hand, oxidized Cyt b_{559} may accept electrons from the acceptor side of PSII (Q_B^-, Q_C, or PQH_2 from the pool) to prevent the formation of damaging singlet oxygen species under acceptor-side photoinhibitory conditions (*e.g.*, under high light conditions).[23, 25-27] In addition, various enzymatic functions of Cyt b_{559} have been proposed, such as superoxide dismutase[28] and PQH_2 oxidase in intact PSII preparations[27, 29-31] and superoxide oxidase and reductase in manganese-depleted PSII preparations[32, 33].

The 2.9-Å–resolution X-ray crystallographic structural model of PSII for the cyanobacterium *Thermosynechococcus elongatus* revealed a newly discovered PQ molecule (Q_C) and its diffusion channel.[34, 35] The head-group of the Q_C molecule is located in a hydrophobic area surrounded by lipid tails, Q_B and a carotenoid molecule. The tail of the Q_C molecule situates in a hydrophobic channel surrounded by the trans-membrane helixes of Cyt b_{559} α and β subunits and PsbJ. The diffusion channel of the Q_C molecule is open toward the internal space of thylakoid membranes.[34, 35] Occupancy of this Q_C site by PQ (or PQH_2) may be involved in exchange of PQ/PQH_2 on the Q_B site from the pool[34, 35] or may modulate the redox potential and reactivity of Cyt b_{559}[34, 36-38]. The existence of this third quinone, Q_C, at only 15 Å from the heme of Cyt b_{559}[17] would support a role for the Cyt b_{559} as a plastoquinol oxidase,

taking electrons from the PQ pool. In fact, a function for Q_C associated with a secondary electron transfer along the Cyt b_{559} in PSII has been proposed[17]. Therefore, Cyt b_{559} may have a role as part of a connecting channel between the PQ pool and Q_B and Q_C sites. However, a recent 1.9-Å–resolution crystal structure model of PSII from *T. vulcanus* showed no occupancy of the Q_C site[39], possibly because of the weak binding of PQ to the site[40]. The function of the Q_C site remains elusive.

Recently, Cyt b_{559} was proposed to have a novel role in modulating photosynthetic light harvesting in PSII reaction centers of cyanobacteria. A spontaneously generated R7Lα Cyt b_{559} mutant of *Synechocystis* sp. PCC 6803 was identified from wild-type (WT) cells grown photoheterotrophically in the presence of 5 mM Glu and 10 μM DCMU.[41] This mutant grew faster than WT cells under photoheterotrophic conditions. In addition, 77 K fluorescence data suggest that the energy transfer from PBs to PSII reaction centers was partially inhibited or uncoupled in this mutant. The results indicated that the R7Lα mutation of Cyt b_{559} may perturb the interaction between the PB and PSII, thereby decreasing the energy delivery from the PB to PSII and thus protecting mutant cells against DCMU-induced photo-oxidative stress. Furthermore, R7Lα and R17Lβ mutant cells of *Synechocystis* sp. PCC 6803 with mutations on the cytoplasmic side of Cyt b_{559} exhibited distinct inhibition of blue-green light-induced NPQ and apparent acceleration of its dark recovery[42]. Thus, the mutations on the cytoplasmic side of Cyt b_{559} may affect the interaction between the phycobilisome and PSII reaction centers, thereby affecting blue-green light-induced NPQ in mutant cells.

To study the physiological function of Q_C and Cyt b_{559} in PSII, we constructed several site-directed mutants of *Synechocystis* sp. PCC 6803 with single point mutations on amino acid residues of Cyt b_{559} and PsbJ located on and near the Q_C site or near the opening of the proposed Q_C diffusion channel in PSII. We discuss

spectroscopic and functional characterizations of these mutant cells and implications for the possible function of the Q_C site in cyanobacteria.

Material and methods

Growth and preparation of Synechocystis sp. *PCC 6803 cells*

WT and mutant *Synechocystis* sp. PCC 6803 cells were photoautotrophically grown in BG-11 medium at 30 °C under light growth conditions with intensity ~30 μE m^{-2} s^{-1} or as described in the figure legends. Cultures were continuously bubbled with sterile, humidified air. Liquid cultures in exponential growth (OD_{730}= 0.7-1.2) were harvested and used for biochemical and functional analysis.

Construction of Cyt b_{559} and PsbJ mutants

The point mutation on amino acid residues of Cyt b_{559} and PsbJ was introduced into the plasmid PAC559EM^R by oligonucleotide-derived mutagenesis as described.[43,44] The DNA sequences of synthetic mutagenic oligonucleotides used in the construction of mutants are in Supplemental Table 1. The mutant was constructed by transforming the mutant plasmid into the host strain (Δ*psbEFLJ*) of *Synechocystis* cells. Mutants were selected on solid media containing the antibiotic Em (0.1 μg mL^{-1}) until their mutated gene was completely segregated. Complete segregation of the mutated gene in these mutant cells was verified by PCR.

Construction of ΔOCP mutants

The *ocp* gene (*slr1963*) was amplified by PCR and cloned into the pMiniT vector (New England Biolabs, USA). A Δ*ocp* plasmid was generated by insertion of a spectinomycin-resistant cassette into the unique *HincII* restriction site of the *slr1963* gene. The Δ*ocp* plasmid was used to transform WT, Cyt b_{559} and PsbJ mutants. ΔOCP

mutants were selected on solid media containing the antibiotics Em (0.1 μg mL^{-1}) and Sp (20 μg mL^{-1}) until their mutated gene was completely segregated. Complete segregation of the mutated gene in these ΔOCP mutant cells was verified by PCR.

Measurement of photosynthetic oxygen evolution

Steady-state rates of oxygen evolution were measured with a Clark-type oxygen electrode (YSI model 5331 oxygen probe) fitted with a water-jacketed cell. Concentrated cells were diluted into growth medium held at 25°C in a stirred, water-jacketed cell. Amounts of 2 mM potassium ferricyanide and 2,6-dichloro-*p*-benzoquinone (DCBQ) were added as an artificial electron acceptor to the BG-11 medium immediately before cells were added. Saturating illumination was provided from both sides of the water-jacketed cell by two fiber-optic illuminators (Dolan-Jenner model MI 150).

Measurement of chlorophyll a fluorescence

Chlorophyll *a* (Chl *a*) fluorescence measurements at 22 ºC were performed with a Dual PAM (pulse-amplitude-modulation) fluorometer (Walz, Germany). The relative PSII content of cells on a chlorophyll basis was estimated from the total yield of variable chlorophyll *a* fluorescence (F_m-F_0) measured in the presence of DCMU and hydroxylamine as described.[42] Experimental conditions for measuring time-dependent flash-induced transients of PSII fluorescence yield and the kinetics of electron transfer from Q_A^- to Q_B in response to a saturating flash given to cells are described in figure legends.

Thermoluminescence (TL) measurements

TL glow curves of *Synechocystis* sp. PCC 6803 cells were measured by using a

home-built apparatus as described.[45-47] Typically, cyanobacterial suspensions were dark-incubated for 3 min at 30 °C, then cooled to 0 °C for 1 min and illuminated at the end of this period with different numbers of saturating single turn-over flashes (separated by 1 s) of white light through an optic fiber. Luminescence emission was recorded while warming samples from 0 °C to 70 °C at a heating rate of 0.5 °C s^{-1}. The instrument was driven by a personal computer with a specially developed acquisition program.[48] Signal analysis and graphical simulation were performed as described.[48]

Absorption and low-temperature fluorescence measurements

Absorption spectra were measured on a PerkinElmer Lambda 35 spectrometer. The optical resolution was 1 nm. All the optical absorption measurements were performed at room temperature with cell suspensions under the same optical density at 730 nm. Fluorescence emission spectra were recorded with a fluorescence spectrometer (Jasco model FP-6500). All fluorescence measurements were performed at 77 K, with cell suspensions at a chlorophyll concentration of 20 µg mL^{-1}. Excitation lights of a λ = 435 nm and a λ = 580 nm were used for exciting chlorophyll *a* (excitation band width 5 nm, emission band width 1 nm) and phycobilisomes (excitation band width 3 nm, emission band width 1 nm), respectively.

Immunoblot detection of OCP

Membrane fractions were obtained by centrifugation of cells broken in a phosphate/citrate buffer to obtain the PB-associated membrane (MP) fraction or MES buffer to obtain the PB-free membrane (M) fraction as described[7]. MP and M fractions equivalent to 2 µg chlorophyll were separated by SDS-PAGE on 12% polyacrylamide/2 M urea gel in a Tris/MES system.[49] Detecting OCP protein involved

incubation with rabbit OCP-specific antisera as the primary antibody[7] and peroxidase-conjugated goat anti-rabbit antibody as the secondary antibody (Sigma). Bands were visualized by using of Western Lightning *Plus*-ECL (PerkinElmer).

Liquid chromatography–mass spectrometry analysis

The relative abundance of PQ and PQH_2 in the WT and mutant cells was determined by ultra-performance liquid-chromatography atmospheric-pressure chemical-ionization quadrupole time-of-flight mass spectrometry (UPLC-APCI-QTOFMS) as described[50].

Biomass determination

Cultures were grown photoautotrophically in the growth chamber at 30 °C under white fluorescent lights (~30 μE m^{-2} s^{-1}). In total, 100 mL of culture were harvested after 126 h and concentrated into 5 mL by centrifugation, then oven-dried in aluminum dishes for 24 h at 105 °C. After cooling to room temperature the dry weight was determined[51].

Results

Growth and photosynthetic characteristics of mutant cells

We constructed several site-directed mutants of Cyt b_{559} and PsbJ with mutations on and near the Q_C site (A16FJ, A16LJ, A16SJ, G19FJ, A20FJ, and V32Fβ of Cyt b_{559}) and near the opening of the proposed Q_C transfer channel (S23Aα, S28Aβ and S28Vβ of Cyt b_{559}) in the model cyanobacterium *Synechocystis* sp. PCC 6803 (Table 1 and Figure 1).[34, 35] Our in silico structural model analysis of the Q_C binding pocket in WT and mutant PSIIs (see Fig. 1S) suggests that substituting Ala16 or Ala20 residues of PsbJ or Val32 residue on the beta-subunit of Cyt b_{559} with bulky phenylalanine (Phe)

may hinder or block the binding of the PQ molecule to the Q_C site of PSII. In contrast, substituting the Ala16 residue of PsbJ with Leu or Ser or substituting Gly19 residue of PsbJ with Phe may have much less effect. In addition, Ser23α and S28β residues of Cyt b_{559} (corresponding to Ser24α and Thr30β residues of Cyt b_{559} in *T. elongatus*) are located near the opening of the proposed Q_C channel[34, 35]. We wondered whether substituting either of these two Ser residues of Cyt b_{559} by Ala might affect the diffusion of the PQ molecules to the Q_C site. Our results showed that all mutant cells grew photoautotrophically like WT cells, and the maximal O_2 evolution rates and estimated PSII content of the mutant and WT cells were similar (Table 1). In addition, the kinetics of electron transfer from Q_A^- to Q_B and Q_B^- were similar between A16FJ, V32Fβ, S23Aα and S28Aβ mutant cells and WT cells (see Fig. 2S), except for a slight increase in a very slow phase in fluorescence decay kinetics for S23Aα mutant cells, which could be attributed to the charge recombination between Q_A^- and PSII electron donors.[52-54] The other mutant cells in Table 1 all showed the normal electron transfer from Q_A^- to Q_B and Q_B^- like WT cells (data not shown). Furthermore, in the presence of DCMU, in response to a saturating flash, the kinetics of charge recombination between Q_A^- and PSII electron donors was similar in mutant and WT cells, as measured by Chl *a* fluorescence (data not shown). Thus, the Mn cluster and Q_A were generally intact in the PSII of mutant cells.

PSII fluorescence yield in the presence and absence of blue actinic light

In cyanobacteria, dark conditions induce state 2 and weak blue light illumination induces state 1. With medium-intensity blue actinic light (~60 μE m^{-2} s^{-1}), the increase in PSII fluorescence yield due to the transition to state 1 was smaller in WT cells (Figure 2A) than in A16FJ, S28Aβ, V32Fβ and S23Aα mutant cells (Figure 2B-E), After this, the gradual decline in WT cells corresponding to the blue-light–induced

NPQ (Figure 2A) was not observed in mutant cells (Figure 2B-E), which indicates no substantial blue-light–induced NPQ. In contrast, G19FJ mutant cells (Figure 2F) showed similar effects as WT cells. In addition, F_o values were slightly higher for dark-adapted mutant than WT cells (A16FJ, 0.15±0.01; S28Aβ, 0.15±0.01; V32Fβ, 0.16±0.01; S23Aα, 0.14±0.01 *vs.* 0.13±0.01) except for G19FJ mutant cells, which showed a slightly lower F_0 value (0.12±0.01) (Figure 2). Figure 3 shows the changes in time-dependent flash-induced PSII fluorescence yield for WT and mutant cells with strong blue actinic light followed by a period of recovery in the absence of actinic light. With 400 μE m^{-2} s^{-1} blue actinic light, WT cells showed a strong quenching in fluorescence yield, and steady-state fluorescence (F_s) levels dropped below the F_o level (Figure 3A). With blue actinic light turned off, the F_m' and F_o' values recovered gradually in the dark. The rate of fluorescence recovery in WT and mutant cells decreased with increasing NPQ amplitude. Quantitative analysis of blue-light-induced NPQ effects for WT and mutant cells are shown in Figure 4. As compared with WT cells, A16FJ, S28Aβ, V32Fβ and S23Aα mutant cells showed lower blue-light-induced NPQ (Figures 3B-E and 4), but G19FJ mutant cells were similar to WT in NPQ (Figure 3F) under the same experimental conditions. A20FJ and S28Vβ mutant cells showed a substantial decrease in the blue-light-induced NPQ, but A16SJ and A16LJ mutant and WT cells showed large quenching (data not shown). Furthermore, with pre-illumination of medium-intensity blue light (~60 μE m^{-2} s^{-1}, 2 min) to trap these cells in State 1 (see Fig. 3S) or with treatment of high osmotic strength solution (0.4 M betaine) to inhibit state transitions (see Fig. 4S), the effects of blue-light-induced NPQ were less for both V32Fβ and S28Aβ mutant cells than WT cells. The results suggest that these mutations in Cyt b_{559} and psbJ have an inhibitory effect on OCP-mediated NPQ in these mutant cells.

Effect of blue-light–induced NPQ in ΔOCP mutant cells

To separate the effects of state transitions from those of OCP-mediated NPQ in these Q_C-site mutant cells, we constructed ΔOCP/WT and ΔOCP/Q_C-site mutant cells. Characterization of the effects of blue-light-induced NPQ in ΔOCP/WT and ΔOCP/Q_C-site mutant cells are in Figure 5. These ΔOCP mutant cells were unable to perform OCP-mediated NPQ under blue-light conditions. With medium-intensity blue actinic light (~60 μE m^{-2} s^{-1}), as compared to ΔOCP/WT cells, ΔOCP/Q_C-site mutant cells showed a significant increase in PSII fluorescence yield because of the enhanced state transition from state 2 to state 1. With 400 μE m^{-2} s^{-1} strong blue actinic light, all ΔOCP/Q_C-site mutant cells did not show any significant effect of fluorescence quenching; ΔOCP/WT cells showed a slight effect of fluorescence quenching that could be attributed to the photoinhibition. The results confirm that the decreased fluorescence observed under strong blue light corresponded to the OCP-dependent NPQ. Furthermore, a recent study demonstrated that under strong blue-green light (>150 μE m^{-2} s^{-1}), state transition stopped as soon as the fluorescence quenching appeared in *Synechocystis* sp. PCC 6803.[55] Thus, OCP-mediated NPQ may inhibit the state transition in *Synechocystis* sp. PCC 6803 (with the OCP) under strong blue light.

Thermoluminescence (TL) emissions from WT and mutant cells

We studied the effect of mutations on the TL emission bands of PSII. The TL emitted at physiological temperatures arises from charge recombination from S_2 with Q_A^- (the Q band) and from $S_{2/3}$ with Q_B^- (the B band). The relative stability of these pairs is reflected by the maximum temperature (t_{max}) for these TL emissions. Previous analysis of cyanobacterial mutants of Cyt b_{559} and Cyt c_{550} revealed that the t_{max} of the B-band is strongly sensitive to structural alterations produced on PSII by these mutations.[42, 56, 57] In this section, we describe the characteristics of the TL bands in

whole cells of the three mutants A16FJ, S28Aβ and V32Fβ and the WT. These mutants were selected for the further analysis because they exhibited distinct phenotypes in Chl fluorescence analysis. Figure 6 compares the B-bands of TL obtained after two flashes in cells from WT and mutant strains. The TL signal of WT can be simulated by 2 components with a t_{max} at 32 ºC and 43 ºC corresponding to B1 ($S_3Q_B^-$) and B2 ($S_2Q_B^-$) bands, respectively. The TL signals of the different mutants were also mathematically fitted and de-convoluted into these two components (see Table 2). In A16FJ, S28Aβ and V32Fβ mutants, the B-band was slightly changed to a lower temperature as compared with the WT. The downshift of the t_{max} of the B-bands indicates a relative decrease in the activation energy of the $S_{2/3}Q_B^-$ charge recombination. We assigned this change to a minor modification of the acceptor side, possibly Q_B side, because B1 ($S_3Q_B^-$) and B2 ($S_2Q_B^-$) change in the same direction. These mutations may have a small effect on the Q_B site (*e.g.*, weakens the binding strength of oxidized plastoquinone), which shifts the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ charge equilibrium to the left and lowers the redox potential (E_m) of Q_B/Q_B^-. After excitation with two flashes, the S_2-related B2 band was the major component for the WT and A16FJ and S28Aβ mutants, whereas the S_3-related B1 band became the main peak-forming component for the V32Fβ mutant. This latter mutation could have a small but significant effect on the Q_B site increasing the dark ratio Q_B^-:Q_B and thus a higher ratio of B1- to B2-band, assuming no changes in the dark S_0:S_1 ratio (25:75). Furthermore, the intensity of the B-band exhibited a typical four-oscillation period with maxima after the 2nd flashes for WT and all mutant cells (data not shown).

Absorption and low-temperature fluorescence measurements

Absorption spectra for WT and mutant cells recorded at room temperature are in Figure 7. The content of chlorophyll *a* and phycobilin, determined by the amplitude of

absorption bands at 685 and 635 nm, respectively, was similar among the WT and A16FJ and S28Vβ mutant cells. V32Fβ mutant cells showed a small increase in ratio of phycobilin to chlorophyll. In addition, polypeptide analysis revealed no difference in composition of PBs between the WT and mutants (data not shown). Furthermore, WT and A16FJ mutant cells did not significantly differ for 77 K-fluorescence emission spectra obtained when the excitation light was 580 nm (absorbed by PBs) or 435 nm (absorbed by Chl *a*) (Fig. 4S, panels A and B, respectively). Our results suggest no significant changes in the energy transfer from the PBs to PSII reaction centers and in PSII:PSI ratio between the WT and A16FJ mutant cells. V32Fβ mutant cells showed small changes in the emission peaks of PSII (at ~693 nm for V32Fβ mutant *vs.* ~695 nm for WT and A16FJ mutant cells) in 77 K-fluorescence emission spectra obtained when the excitation light was 580 nm (absorbed by PBs) and a slight increase in the intensity of emission at ~688 nm in 77 K-fluorescence emission spectra obtained when the excitation light was 435 nm (absorbed by Chl *a*) (Fig. 5S, panels A and B, respectively). The results indicate a very subtle change in the energy transfer from the PBs to PSII reaction centers in V32Fβ mutant cells, with no significant changes in PSII:PSI ratio between the WT and V32Fβ mutant cells.

Immunoblot detection of OCP

A previous study demonstrated the strong interaction of OCP with thylakoids by detecting OCP-GFP proteins in MP (PB-associated) and M (PB-free) membrane preparations.[7] Most of the OCP-GFP proteins co-precipitated with the membrane fractions.[7] We found higher OCP protein level (about two-fold) in MP than M membrane preparations for the WT and mutant thylakoid membranes (Figure 8). In addition, the level of OCP in thylakoid membranes (both MP and M membrane preparations, see Figure 8) and soluble protein fractions (data not shown) was similar

among the WT and A16FJ, V32Fβ and S28Vβ mutant cells. Therefore, A16FJ, V32Fβ and S28Vβ mutations did not alter the interaction between OCP and thylakoids. Furthermore, the reduced level of OCP-induced NPQ in mutant cells was not due to lack of OCP.

The redox state of the PQ pool

State transitions in cyanobacteria are known to be affected by the redox state of the PQ pool. To determine the possible change in the redox state of the PQ pool in mutant cells, the UPLC-APCI-QTOFMS method was used to measure the relative abundance of PQ and PQH_2 in WT and mutant cells.[42] This method was developed for rapid and simultaneous profiling of prenylquinones in plant tissues by UPLC-MS.[50] Previously, we used this technique to demonstrate that the redox state of the PQ pool in the dark was more reduced in R7Eα and R17Lβ Cyt b_{559} mutant than WT cells[42]. The mean PQ/PQ_{tot} ratios were 0.63±0.04, 0.69±0.04, 0.67±0.07, 0.69±0.06 (n=3) for the WT, A16FJ, V32Fβ and S28Aβ mutant cells, respectively (Figure 9). Thus, the redox state of the PQ pool in the dark was similar in the mutant and WT cells.

Photosynthetic growth rate and biomass analysis of mutant cells

Photosynthetic growth rate was greater in A16FJ, V32Fβ and S28Aβ mutant than WT cells under our normal growth conditions (~30 μE m^{-2} s^{-1}) (Figure 10). The mean ± SE doubling-time growth rate was 17.7±0.2, 16.3±0.2, 16.0±0.2, and 16.5±0.7 (n=3-4) for WT, A16FJ, V32Fβ and S28Aβ mutant cells, respectively (Table 3). In addition, biomass concentration calculated for 5-day cultures of A16FJ, V32Fβ and S28Aβ mutant cells was about 1.31-, 1.42-, and 1.38-fold, respectively, higher than for WT cells under the same conditions (Table 3).

Discussion

Mutations on Cyt b_{559} and PsbJ alter the regulation of photosynthetic light harvesting

In this work, we report spectroscopic and functional characterizations of several Cyt b_{559} and PsbJ mutant cells with mutations on and near the Q_C site and near the opening of the proposed Q_C transfer channel in PSII in the cyanobacterium *Synechocystis* sp. PCC 6803. One of the distinct phenotypes in mutant cells was the enhanced transition from state 2 to state 1 during medium-intensity blue-light illumination (see Figures 2). A similar enhanced state transition was observed in ΔOCP/Q_C-site mutant cells as compared to ΔOCP/WT cells (see Figure 5). State transitions in cyanobacteria responded to changes in redox state of the PQ pool.[1-5, 58] The redox state of the PQ pool was similar in A16FJ, V32Fβ and S28Aβ mutant cells and WT cells in the dark (see Figure 9). Therefore, the enhanced state transition in these mutant cells under our experimental conditions was not due to the more reduced PQ pool in the dark as compared to WT cells. In addition, the cytoplasmic side of Cyt b_{559} is located within the predicted contact sites in PSII for the APC core complex of the PB.[59] Our results agree with previous mutagenesis studies of *Synechocystis* sp. PCC 6803 suggesting that mutations on the cytoplasmic side of Cyt b_{559} may alter the interaction between the PB and PSII reaction centers, thereby affecting the regulation of photosynthetic light harvesting.[41, 60] State transitions in cyanobacteria are affected by the redox state of the PQ pool, so determining whether the Q_C site may be involved in their regulation is of interest. From our in silico structural analysis of the Q_C binding pocket in WT and mutant PSIIs (see Fig. 1S), replacing Ala16 or Ala20 residues of PsbJ or Val32 on the beta-subunit of Cyt b_{559} with bulky Phe may hinder or block the binding of the PQ molecule to the Q_C site of PSII. In contrast, replacing the Ala16 residue of PsbJ with Leu or Ser, or replacing the Gly19 residue of PsbJ with Phe would have much less effect. Interestingly, our fluorescence results showed that

the enhanced transition from state 2 to state 1 was stronger for A16FJ, A20FJ and V32Fβ mutants but much lower in A16LJ, A16SJ and G19FJ mutant cells than WT cells during medium-intensity blue-light illumination. Thus, our results are consistent with the possible involvement of the Q_C site in regulating the state transition in cyanobacteria. In addition, transition from state 2 to state 1 was also greater for S23Aα, S28Aβ and S28Vβ Cyt b_{559} mutant cells than WT cells during medium-intensity blue-light illumination. Because Ser23α and S28β residues of Cyt b_{559} (corresponding to Ser24α and Thr30β residues of Cyt b_{559} in *T. elongatus*) are located near the opening of the proposed Q_C channel[34, 35], replacing these Ser residues with Ala might hinder the diffusion of the PQ molecules to the Q_C site in mutant PSIIs. Alternatively, because these two Ser residues may form hydrogen bond interactions with the main-chain carbonyl group of Cyt b_{559}[34, 35], substituting either of these Ser residues with Ala may disrupt the corresponding hydrogen bond interaction, which may alter the conformation of Cyt b_{559} and thus cause the above effect.

The other distinct phenotype of these mutant cells is the weakened effect of blue-light–induced NPQ under strong blue-light conditions (Figure 3 and Table 1). Previous studies have demonstrated that the activated red form of OCP interacts with the PB and induces NPQ in cyanobacteria.[6, 7, 18] The interaction site for the OCP was proposed to be on one of the central APC disks of the base cylinders.[11, 14, 17, 18, 61] We found that the protein level of OCP was similar in thylakoid membranes from the WT and A16FJ, V32Fβ and S28Vβ mutant cells (Figure 8). Therefore, the attenuated effect of OCP-induced NPQ in these mutant cells was not due to lack of OCP. In addition, our previous study showed that the R7Lα and R17Lβ mutation on the cytoplasmic side of Cyt b_{559} significantly inhibited blue-light-induced NPQ and accelerated its dark recovery[42]. These results support these mutations on Cyt b_{559} and PsbJ possibly inducing a subtle structural modification on the cytoplasmic site of PSII

that affects the interaction of the APC core complex with OCP, thereby altering OCP-induced NPQ in cyanobacteria.

Currently the mechanism of OCP-induced NPQ seems to depend on only PBs, OCP and FRP.[7, 13] In addition, previous studies demonstrated that OCP-induced NPQ in cyanobacteria is not directly affected by changes in transthylakoidal ΔpH or the redox state of the PQ pool but rather light irradiance and quality.[7] Furthermore, previous studies with DNA microarray analysis showed significantly increased transcripts of the OCP gene under high light or DBMIB treatments.[62] The OCP-induced NPQ in cyanobacteria is likely influenced by the increased OCP protein level by upregulation of the OCP gene during high light and other stress conditions.[62] Thus, the OCP-induced NPQ in cyanobacteria seems less likely to be mediated by the Q_C site in response to the change in redox state of the PQ pool. However, our results show that mutations of Cyt b_{559} and PsbJ on and near the Q_C site and the opening of the proposed Q_C transfer channel significantly weaken the effect of OCP-induced NPQ in *Synechocystis* sp. PCC 6803. Future research should investigate whether the Q_C site in PSII plays a structural role or a complementary functional role in modulating OCP-induced NPQ in cyanobacteria.

Other proposed functions of the Q_C site

Previous studies proposed that occupancy of the Q_C site by PQ (or PQH_2) may be involved in exchange of PQ/PQH_2 on the Q_B site from the pool.[34, 35] Because our mutants were constructed on residues of Cyt b_{559} and PsbJ on and near the Q_C and near the opening of the proposed Q_C transfer channel, replacing these residues with bulky phenylalanine may hinder or block the diffusion of the PQ molecule through the channel. TL analysis showed that V32Fβ and S28Aβ mutant cells had a small but significant effect on the acceptor side of PSII (Q_B^-), but our mutant cells -- A16FJ,

V32Fβ, S28Aβ, G19FJ, A20FJ -- showed normal activity of photosynthetic water oxidation and kinetics of electron transfer from Q_A to Q_B and Q_B^- (Table 1). Therefore, our results suggest that the Q_C site and Q_C channel may not play an important role in the exchange of PQ/PQH_2 on the Q_B site from the pool under normal growth conditions. Moreover, a previous study reported that the oxidation of Q_A^- via forward electron flow to Q_B is slower by about 40% in the *psbJ* stop mutant (lack of PsbJ) of *Synechocystis* sp. PCC 6803.[63] This study suggested that PsbJ is important to ensure the forward electron transfer from Q_A via Q_B to the PQ pool. In contrast, most of our Q_C-site mutant cells in Table 1 showed normal electron transfer from Q_A to Q_B as compared to WT cells.

Several authors have proposed that the occupancy of the Q_C site by PQ (or PQH_2) may modulate the redox potential Cyt b_{559} and mediate the redox equilibration between Cyt b_{559} and the PQ pool.[34-38] In addition, previous studies indicated that Cyt b_{559} may function as a PQH_2 oxidase to keep the PQ pool and acceptor-side of PSII oxidized in the dark, which prevents the formation of damaging reactive oxygen species from the acceptor-side photoinhibition.[22, 23, 27, 29-31, 33, 64] The F26Sβ Cyt b_{559} tobacco mutant, R7Eα and R17Lβ Cyt b_{559} mutant cells of *Synechocystis* sp. PCC 6803 and R18Sα Cyt b_{559} mutant cells of *T. elongatus* showed largely reduced PQ pools, altered redox-potential forms of Cyt b_{559}, and high susceptibility to light stress.[30,34,40,55] A defect in PQH_2 oxidase activity of Cyt b_{559} due to altered redox-potential forms of Cyt b_{559} in these mutant strains could explain their high susceptibility to strong light and greatly reduced PQ pools. However, our results showed that the redox state of the PQ pool in the dark was similar in the A16FJ, S28Aβ and V32Fβ mutant and WT cells. Thus, we detected no apparent defect in PQH_2 oxidation in the dark in these mutant cells. In addition, these Q_C site mutant cells showed normal oxygen-evolution activity and PSII stability under our experimental conditions.

Therefore, these Q_C site mutations (*e.g.* A16FJ, S28Aβ and V32Fβ) may not significantly perturb the photoprotective function of Cyt b_{559} in the mutant cells. Further studies are required to verify this discrepancy and to determine the exact physiological function of the Q_C site in PSII.

Modulating photoprotection in cyanobacteria may improve photosynthetic growth and biomass production

Interestingly, we found greater photosynthetic growth rate and biomass production for A16FJ, V32Fβ and S28Aβ mutant than WT cells (about 1.1-fold and 30-40%, respectively) under our normal growth conditions. These mutant cells showed significantly enhanced transition from state 2 to state 1 during medium-intensity blue-light illuminations and weakened effects of OCP-induced NPQ during high-intensity blue-light illumination. Therefore, the increase in photosynthetic growth rate and biomass production in A16FJ, V32Fβ and S28Aβ mutant cells could be attributed to decreased dissipation of wasteful energy from PBs and improved photosynthesis efficiency in mutant rather as compared with WT cells. Our results support manipulation of photoprotection possibly improving photosynthesis and biomass production of cyanobacteria.[65-67] In natural living environments, cyanobacteria require diverse photo-protective mechanisms to ensure their survival under various stress conditions (light, temperature, nutrients etc.). However, in controlled environments, such as in growth chambers and photobioreactors, attenuating photoprotection may improve photosynthesis efficiency and biomass production of cyanobacteria.

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Supporting Information Available (http://pubs.acs.org)

Figure 1S. In silico structural models of the Q_C binding pocket in wild-type (WT) and mutant photosystem II of *Synechocystis* sp. PCC6803.

Figure 2S. Kinetics of electron transfer from Q_A^- to Q_B and Q_B^- in response to a saturating flash given to WT and mutant cells.

Figure 3S. Time-dependent, flash-induced PSII fluorescence yield for (A) WT, (B) V32Fβ, and (C) S28Aβ mutant cells [pre-illuminated with medium blue light (~60 μE m^{-2} s^{-1}) for 2 min] with and without strong blue actinic light. The intensity of strong blue light was ~400 μE m^{-2} s^{-1}.

Figure 4S. Time-dependent, flash-induced PSII fluorescence yield for (A, B) WT, (C, D) V32Fβ, and (E, F) S28Aβ mutant cells with and without strong blue actinic light. The intensity of strong blue light was ~400 μE m^{-2} s^{-1}. (B), (D) and (F) were treated with 0.4M betaine to inhibit state transition. The cells were grown under light conditions with intensity 80-90 μE m^{-2} s^{-1}.

Figure 5S. 77 K-fluorescence emission spectra for WT, A16FJ and V32Fβ mutant cells.

Table 1S. The DNA sequences for synthetic mutagenic oligonucleotides.

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Table 1. Summary of the properties of wild-type (WT) and mutant cells

Strain	Blue-light–induced NPQ effect[a]	O_2 evolution[b] (% of WT)	Estimated PSII content[c] (% of WT)
WT	+	100 ± 6[d]	100 ± 10
A16FJ	-	97 ± 5	85 ± 8
A16LJ	+	87 ± 6	93 ± 1
A16SJ	+	93 ± 6	86 ± 3
G19FJ	+	79 ± 6	98 ± 5
A20FJ	-	93 ± 10	103 ± 2
S23Aα	-	83 ± 1	104 ± 2
S28Aβ	-	79 ± 5	100 ± 1
S28Vβ	-	86 ± 5	87 ± 8
V32Fβ	-	95 ± 2	105 ± 6

[a] "+", normal and "-", significantly inhibited blue-light–induced NPQ effect.

[b] The mean± SD O_2 evolution rate of WT cells was 509 ± 30 μmol O_2 mg Chl^{-1} h^{-1}.

[c] Estimated from the variable fluorescence yield ($F_m - F_o$).

[d] Data are mean ± SD from 3-4 independent experiments.

Table 2. The peak position of the B bands from the thermoluminescence curves of WT and mutant cells

	WT	A16FJ	S28Aβ	V32Fβ
B1 band	32 ± 2 °C	31 ± 1 °C	28 ± 2 °C	28 ± 2 °C
B2 band	43 ± 1 °C	39 ± 1 °C	39 ± 2 °C	37 ± 1 °C

t_{max} values were obtained for B1 and B2 bands for WT and mutant cells from the de-convolution of signals after two flashes and statistical analysis. For further details see the Material and Methods section.

Table 3. Photosynthetic growth rates and biomass production in WT and mutant cells under ~30 μE m^{-2} s^{-1} light intensity.

Strain	Doubling time (hour)	Biomass concentration (mg/ml)
WT	17.7 ± 0.2	0.213 ± 0.024
A16FJ	16.3 ± 0.2	0.275 ± 0.037
S28Aβ	16.5 ± 0.7	0.294 ± 0.026
V32Fβ□	16.0 ± 0.2	0.303 ± 0.036

Cultures were grown in the growth chamber at 30°C in ambient air and under continuous illumination (30 μE m^{-2} s^{-1}). Photosynthetic growth rates were measured (with OD 730) in the exponential phase of growth. Biomass concentrations of cultures were measured after about 126 hours of growth. Data are expressed as a mean ± SE from 3-4 independent experiments.

Figure Legends

Figure 1. Location of Q_C, the heme group of Cyt b_{559}, and amino-acid residues of Cyt b_{559} and PsbJ in the structural model of PSII of *Synechocystis* sp. PCC6803. This model was built on the basis of the crystal structure of PSII from *Thermosynechococcus elongatus* at 2.9-Å resolution (PDB ID: 3BZ1) by using the protein structure homology-modeling server (SWISS-MODEL). The figure was created with PyMol (http://www.pymol.org).

Figure 2. Time-dependent flash-induced PSII fluorescence yield for WT (A), A16FJ (B), S28Aβ (C), V32Fβ (D), S23Aα (E), and G19FJ mutant cells (F) with and without medium-intensity blue actinic light. The light intensity was ~60 μE m^{-2} s^{-1}. AL on and off, actinic light on and off, respectively.

Figure 3. Time-dependent flash-induced PSII fluorescence yield for WT (A), A16FJ (B), S28Aβ (C), V32Fβ (D), S23Aα (E), and G19FJ mutant cells (F) with and without strong blue actinic light. The light intensity was ~400 μE m^{-2} s^{-1}. The other conditions were same as in Figure 2. AL on and off, actinic light on and off, respectively.

Figure 4. Quantitative analysis of NPQ during time-dependent, flash-induced PSII fluorescence yield measurement of WT and mutant cells with and without strong blue actinic light. The light intensity was ~400 μE m^{-2} s^{-1}. The experimental conditions were as in Figure 3. Data are the mean ± SD of 6-8 independent experiments.

Figure 5. Time-dependent flash-induced PSII fluorescence yield for ΔOCP (A,E), ΔOCP /A16FJ (B,F), ΔOCP/V32Fβ (C,G), and ΔOCP/S28Aβ mutant cells (D,H) with and without blue actinic light. The light intensity was (A) to (D) ~60 μE m^{-2} s^{-1} and (E) to (H) ~400 μE m^{-2} s^{-1}. The other conditions were as in Figure 2.

Figure 6. Thermoluminescence glow curves (B bands) of the WT and mutant cells recorded after two flashes. The dashed lines represent the simulation components (B1-band and B2-band) corresponding to the best fit (see Material and Methods).

Figure 7. Absorption spectra for WT and mutant cells recorded at room temperature. Each spectrum was normalized at 750 nm and then offset by 0.05 absorbance units.

Figure 8. Immunoblot detection analysis of OCP in phycobilisomes-associated (MP) and phycobilisomes free (M) membrane preparations for WT, A16FJ, S28Vβ and

V32Fβ thylakoid membranes. Each lane contained 2 μg chlorophyll.

Figure 9. The redox state of the plastoquinone pool (PQ) in the WT, A16FJ and V32Fβ mutant cells measured by UPLC-APCI-QTOFMS method. Samples were incubated in darkness for 10 min. The mean ± SD ratios of PQ/PQ_{tot} were 0.63±0.04, 0.69±0.04, 0.67±0.07, 0.69±0.06 for the WT, A16FJ, V32Fβ and S28Aβ mutant cells, respectively.

Figure 10. Photosynthetic growth curves of WT and mutant cells under ~30 μE m^{-2} s^{-1} light intensity. Data are mean ± SD of 3 independent experiments.

Graphic for the Table of Contents

Mutations of cytochrome b_{559} and PsbJ on and near the Q_C site in photosystem II influence the regulation of short-term light response and photosynthetic growth of the cyanobacterium *Synechocystis* sp. PCC 6803

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