Supporting Information

Structural Coupling of an Arginine Side Chain with the Oxygen Evolving Mn_4Ca Cluster in Photosynstem II As Revealed by Isotope-Edited Fourier Transform Infrared Spectroscopy

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1. Experimental Procedures

Culture of Cyanobacterial cells. *Synechocystis* sp. PCC 6803 was grown in the BG-11 medium[1] containing 5 mM TES buffer (pH 8.2) and 5 mM glucose with a light intensity of 30 μmole photon m^{-2} s^{-1}. The temperature was maintained at 25°C and air was supplied through an air filter (Millex, Millipore, USA).

Construction of an arginine-requiring strain. The Arg-requiring strain (designated as ΔArgH) of *Synechocystis* sp. PCC 6803 was generated by deletion of a putative gene encoding L-argininosuccinate lyase (*slr1133, argH*) using a strain in which a hexa-histidine tag was added to the C-terminus of CP47 (designated as WT).[2] We constructed a plasmid vector pZArgH::Sp(−) in which *argH* was replaced with a spectinomycin/streptomycin resistance gene cassette inserted between the upstream (0.9 kb) and downstream (1.0 kb) regions of *argH* using a cloning vector pZErO-2.1 (Invitrogen, USA). Combinations of PCR primer sets, P1/P2

(P1:5′-GAATTCTTAACCACCTCAACCGGG-3′, P2:5′-GTCGACCTTCAAAACGATCGCTCCAG-3′) and P3/P4 (P3:5′-GTCGACCCTGGCGGAATGGCAAGC-3′, P4:5′-GGATCCCAGTGTAGAAGCGATCGC-3′), were used for amplification of the upstream and downstream regions, respectively (Figure S1A). WT was transformed using the pZArgH::Sp(−), and the deletion strain was selected on agar plates containing 5 mM L-Arg·HCl, 0.3% sodium thiosulfate, 5 μg/ml spectinomycin, and 5 μg/ml chloramphenicol as supplemental elements. A large-scale culture (up to 16 L) was carried out in a liquid medium containing 1 mM L-Arg·HCl and the antibiotics at the same concentrations as used for the agar plates. For isotope labeling of Arg in cells, L-[$\eta_{1,2}$-$^{15}N_2$]Arg·HCl or L-[ζ-^{13}C]Arg·HCl (Cambridge Isotope Laboratories Inc., USA; 99 atom % ^{15}N or ^{13}C) was used instead of unlabled L-Arg·HCl.

PCR and Southern hybridization analysis. The *argH* deletion was confirmed by PCR and Southern hybridization analyses. PCR analysis was executed by amplification of the region between the upstream and downstream of *argH* using the primer set P1/P4 (Figure S1A) using genomic DNA from WT and ΔArgH strains and the pZArgH::Sp(−) as templates. Southern hybridization was performed using AlkPhos Direct Labeling and Detection System (GE Healthcare, UK) as the manufacturer's instructions. Genomic DNA from WT and ΔArgH strains was digested with a restriction enzyme *Hin*dIII. The digested DNA was separated by agarose gel electrophoresis, and transferred onto a nylon membrane (Hybond-N+, GE Healthcare). As a probe, a PCR product (0.9 kb) amplified using the primer set P1/P2 (Figure S1A) was labeled with thermostable alkaline phosphatase.

Chemiluminescent detection was carried out using CDP-*Star* Detection Reagent (GE Healthcare).

Isolation of PSII core complexes and analysis of the subunit composition. PSII complexes were isolated as described previously.[2,3] Briefly, cells were suspended in a 50 mM MES-NaOH buffer (pH 6.0) containing 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 25% (w/v) glycerol, and were then broken using glass beads. Thylakoid membranes were collected by centrifugation and resuspended in the above buffer (1 mg Chl/ml). PSII core complexes were solubilized from the thylakoid membranes by the treatment of 0.8% (w/v) dodecyl-β-D-maltoside (DM) and subsequent incubation at 4°C for 20 min in the dark, and then purified by Ni^{2+}-affinity column chromatography (ProBond^{TM} resin, Invitrogen, USA). The purified PSII complexes were concentrated and dissolved in the same buffer containing additional 0.04% DM. The subunit composition of the PSII core complexes was analyzed by SDS-PAGE; the stacking and running gels contained 6% and 18–24% acrylamide, respectively.[4] Proteins corresponding to 2 μg Chl *a* were loaded in each lane. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. Western blotting against the PSI proteins was performed as previously described.[3]

Measurement of oxygen-evolution activity. Oxygen-evolution activity was measured using a Clark-type oxygen electrode (Rank Brothers, England). PSII core complexes were suspended in a 50 mM Mes-NaOH buffer (pH 6.0) containing 1 M sucrose, 25 mM $CaCl_2$, and 10 mM NaCl. The Chl content of samples was adjusted to ~2 μg/ml. Potassium ferricyanide (final concentration: 4 mM) was used as an electron acceptor.

Measurements of absorption and fluorescence spectra. Absorption and

fluorescence spectra were measured using a Hitachi 557 spectrophotometer and a Hitachi 850 spectrofluorometer, respectively.[2,3] When fluorescence spectra were measured at a cryogenic temperature (−196°C), polyethylene glycol (average molecular weight: 3,350; final concentration: 15% (w/v)) was added to the sample. The wavelength-dependent sensitivity of the fluorometer was corrected using a sub-standard lamp with a known radiation profile.

FTIR measurements. Light-induced FTIR difference spectra were measured as previously described.[3,5] The PSII core complexes suspended in a 10 mM Mes-NaOH (pH 6.0) buffer containing 5 mM NaCl, 5 mM $CaCl_2$, and 0.03% DM was concentrated to about 2.3 mg Chl/ml using Microcon-100 (Amicon). An aliquot of the sample suspension (10 μl) was mixed with 1 μl of 100 mM potassium ferricyanide and dried on a CaF_2 plate (25 mm in diameter) under N_2 gas in an oval shape (6 × 9 mm). The sample was moderately hydrated by placing 2 μL of a 40% (v/v) glycerol/H_2O solution in a sealed IR cell without touching the sample.[5] The sample temperature was kept at 10 ˚C by circulating cold water in a copper holder. Flash-induced FTIR difference spectra were measured using a Bruker IFS-66/S spectrophotometer and a Q-switched Nd:YAG laser (Quanta-Ray GCR-130; 532 nm; ~7 ns FWHM). For S_2/S_1 measurements, after dark adaptation for 20 min, single-beam spectra (50-s accumulation) were recorded before and after a single saturating flash. The measurement cycle including 20-min dark adaptation was repeated 24 times for one sample and the data of two samples were averaged.

FTIR spectra of L-Arg·HCl (unlabeled, $\eta_{1,2}$-$^{15}N_2$-labeled, and ζ-^{13}C-labeled) and ethylguanidine·HCl in aqueous solutions (0.5 M) were measured at room temperature

between BaF_2 plates. A piece of aluminium foil (1 mm x 1 mm) was also sandwiched as a spacer. All FTIR spectra were recorded with a resolution of 4 cm^{-1}.

2. Characterization of the ΔArgH strain and isotope-labeled PSII core complexes

Property of the Arg-requiring strain. The deletion of *argH* in ΔArgH strain was confirmed by PCR and Southern hybridization analyses. Figure S1B shows a PCR product using the primer set of P1/P4 (Figure S1A). The bands were found at 3.1 and 4.0 kb in WT and ΔArgH, respectively. This size shift corresponds to the extension by the replacement of *argH* with an antibiotic resistance cassette (Figure S1A). On the other hand, Southern hybridization data exhibited distinct fragments of 3.7 and 2.6 kb caused by relocation of the restriction site in WT and ΔArgH, respectively (Figure S1C). No band was found at 3.7 kb in the ΔArgH mutant, although the band was seen in WT even after 20-fold dilution. These results indicate complete deletion of *argH* in the ΔArgH strain.

Figure S1D shows the Arg-dependent growth of the ΔArgH strain. The ΔArgH strain that was supplemented by external Arg grew at a rate similar to WT (the doubling time of ΔArgH was 17, 16, and 15 h in the presence of 1, 3, and 5 mM Arg, respectively, while that of WT was 15 h), whereas it did not grow in the absence of Arg (Figure S1D). All of the above results indicate that the Arg-requiring strain of *Synechocystis* was successfully generated. The observation of no growth in the absence of external Arg (Figure S1D, triangles) indicates that ΔArgH cells cultured with isotope-labeled Arg contain basically no unlabeled Arg.

Absorption and fluorescence spectra of the ΔArgH strain. Absorption spectra of ΔArgH cells with and without isotope labeling measured at room temperature were compared with the spectrum of WT cells (Figure S2A). Spectral features of ΔArgH were virtually the same as those of WT except for a slight increase in the carotenoid band at ~480 nm. Fluorescence spectra by 435 and 600 nm excitation measured at −196°C showed three bands at 686, 695, and 724 nm, of which the former two bands arise from PSII and the latter band arises from PSI, in all the samples (Figure S2B). The composition of PSII and PSI was not significantly modified by ΔArgH mutation and Arg isotope labeling, although ΔArgH cells seem to have a slightly higher PSII content than WT cells. Absorption spectra of the purified PSII core complexes (Figure S3A) also showed a slight increase in the carotenoid absorption at ~480 nm in [$\eta_{1,2}$-$^{15}N_2$]Arg and [ζ-^{13}C]Arg-labeled ΔArgH. The fluorescence spectra by 435 nm excitation measured at −196°C showed two bands at 686 and 695 nm in all samples (Figure S3B). Overall, these results indicate that the optical properties of the PSII complexes from ΔArgH cells are very similar to those of WT PSII.

SDS-PAGE analysis of PSII core complexes. Polypeptide composition of the PSII complexes was examined by SDS-PAGE (Figure S4). The bands of major PSII polypeptides were clearly resolved in the 30–50 kDa region in all of the four samples; the PAGE pattern of thylakoid membranes of WT was clearly different from that of the PSII complexes. All of the PSII complexes from WT, unlabeled ΔArgH, and [$\eta_{1,2}$-$^{15}N_2$]Arg- and [ζ-^{13}C]Arg-labeled ΔArgH showed bands due to CP47, CP43, D1, D2, PsbO, PsbU,

PsbV, PsbE and PsbF, and there were no significant differences among these core proteins. Only the difference is a band at 14 kDa that was increased in the PSII complexes from [$\eta_{1,2}$-$^{15}N_2$]Arg- and [ζ-^{13}C]Arg-labeled ΔArgH. This band was assignable to the PsbQ-like protein (Sll1638) in analogy to the previous report.[6] The PSI components were less than a detection limit of Western blotting using anti-PsaA/B antibodies (Figure S4, bottom panel).

O_2 evolution activity. The O_2 evolution activities of cells and PSII complexes are shown in Table S1. The O_2 activities of unlabeled, [$\eta_{1,2}$-$^{15}N_2$]Arg-labeled, and [ζ-^{13}C]Arg-labeled ΔArgH cells, were 540 ± 20, 440 ± 20 and 480 ± 20 μmol (mg Chl)$^{-1}$ h^{-1}, respectively, which were comparable to 400 ± 10 μmol (mg Chl)$^{-1}$ h^{-1} of WT cells. The activities of isolated PSII complexes were 2690 ± 100, 2370 ± 90 and 1750 ± 20 μmol (mg Chl)$^{-1}$ h^{-1} for unlabeled, [$\eta_{1,2}$-$^{15}N_2$]Arg-labeled, and [ζ-^{13}C]Arg-labeled ΔArgH, respectively, in comparison with 2740 ± 70 μmol (mg Chl)$^{-1}$ h^{-1} for the WT PSII complexes. These results indicate that the isolated PSII core complexes all retained high O_2 evolution activities.

3. FTIR spectra in the 1800–1100 cm^{-1} region

Figure S5 shows the 1800–1100 cm^{-1} region of the S_2/S_1 FTIR difference spectra of unlabeled (a, black line) and [$\eta_{1,2}$-$^{15}N_2$]Arg-labeled (a, red line) PSII complexes of ΔArgH, together with their double difference spectrum (unlabeled-minus-labeled; b). Also, FTIR spectra in the same region of unlabeled (c, black solid line) and $\eta_{1,2}$-$^{15}N_2$-labeled (c, red line) Arg·HCl and of ethylguanidine·HCl (c, black dotted line) in

aqueous solutions are presented together with the unlabeled-minus-$\eta_{1,2}$-$^{15}N_2$-labeled difference spectrum of Arg·HCl (d). Corresponding spectra of [ζ-^{13}C]Arg were presented in Figure S6. It was revealed that virtually no bands of the S_2/S_1 spectra in the 1550–1100 cm^{-1} region were sensitive to [$\eta_{1,2}$-$^{15}N_2$]Arg and [ζ-^{13}C]Arg labeling of PSII (Figures S5 and S6, a and b). This observation is reasonable because there is no strong bands due to a guanidinium group in this region as shown in the spectrum of ethylguanidine·HCl (Figures S5c and S6c, dotted lines) and the difference spectra between isotope-labeled ($\eta_{1,2}$-$^{15}N_2$ and ζ-^{13}C) and unlabeled Arg·HCl in solutions (Figures S5d and S6d). Only a weak signal was detected at 1187/1168 cm^{-1} in the unlabeled-minus-$\eta_{1,2}$-$^{15}N_2$-labeled difference spectrum of Arg·HCl (Figure S6d) due to the low-frequency C-N stretching mode as previously reported by Braiman et al.[7] However, the intensity of this signal was more than 30 times smaller than the strong bands in the 1700–1600 cm^{-1} region (Figures S6d). Thus, the corresponding bands around 1175 cm^{-1} in the S_2/S_1 difference spectrum may not be detectable because of their weak intensities.

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Table S1: O_2 evolution activities (μmol O_2 (mg Chl)$^{-1}$ h^{-1}) of cells and purified PSII complexes

samples	cells	PSII
WT	400 ± 10	2740 ± 70
unlabeled ΔArgH	540 ± 20	2690 ± 100
[$\eta_{1,2}$-$^{15}N_2$]Arg-labeled ΔArgH	440 ± 20	2370 ± 90
[ζ-^{13}C]Arg-labeled ΔArgH	480 ± 20	1750 ± 20

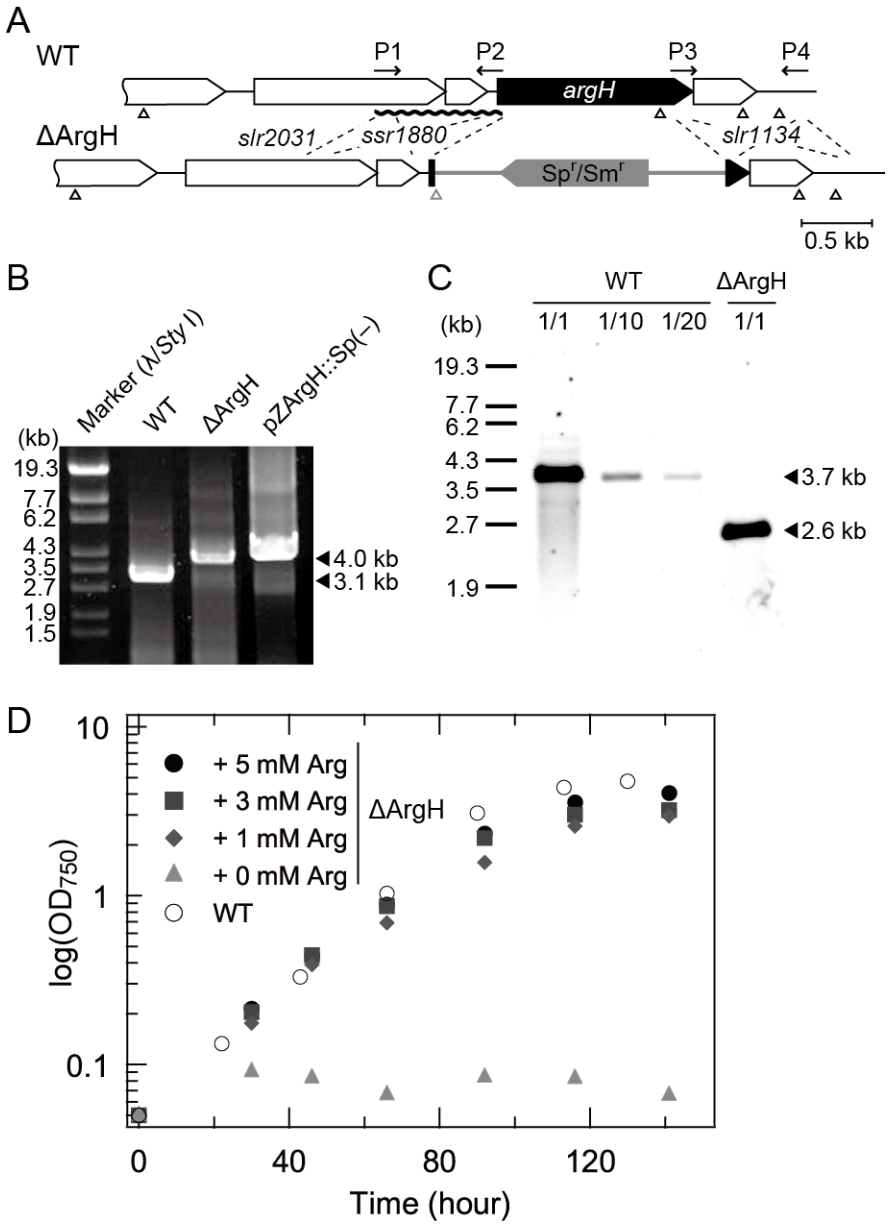

Figure S1: Genotype and phenotype of the arginine-requiring strain (ΔArgH) of *Synechocystis* sp. PCC 6803. (A) Schematic diagram of genomic DNA from WT and ΔArgH. Primers used for PCR are indicated by arrows. Open triangles represent the restriction sites for *Hin*dIII. A region corresponding to a probe is indicated by a wavy line. (B) PCR analysis. (C) Southern hybridization analysis. (D) Growth curves of WT and ΔArgH cells. See text for details.

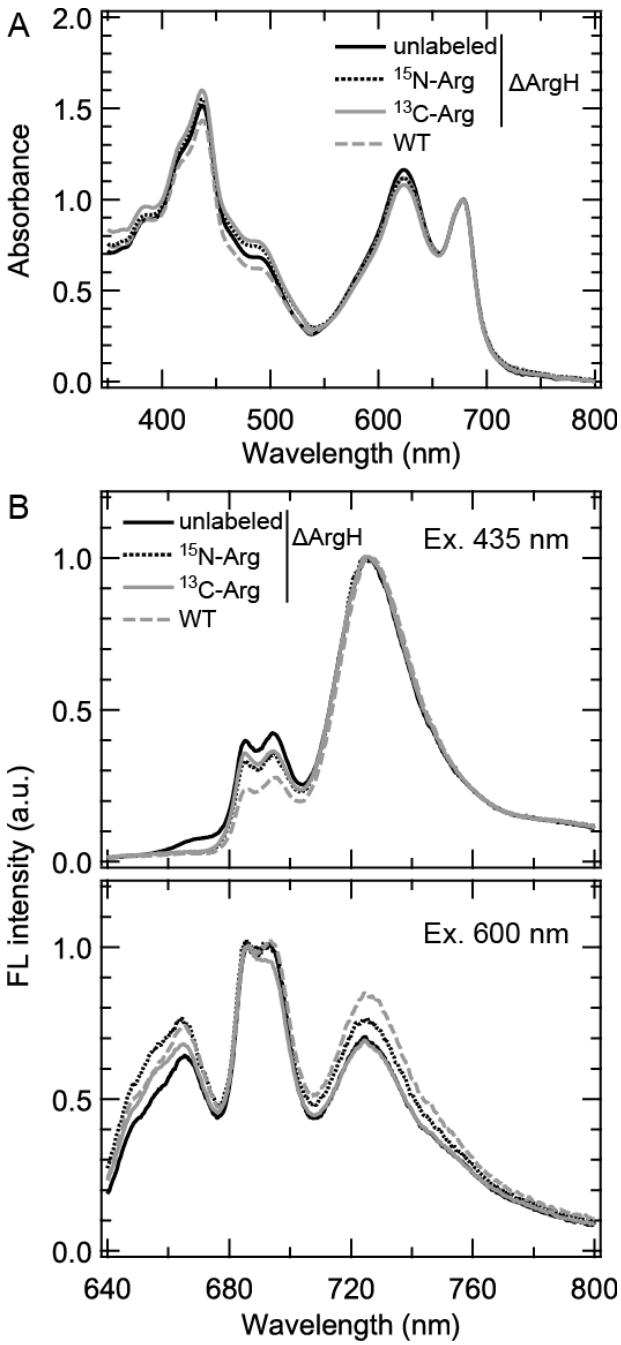

Figure S2: Absorption (A) and fluorescence (B) spectra of WT and ΔArgH cells. Absorption and fluorescence spectra were measured at room temperature and −196°C, respectively. The spectra were normalized at the 678-nm peak in panel A and at the 725-nm (435 nm ex.) or 686-nm (600 nm ex.) peak in panel B.

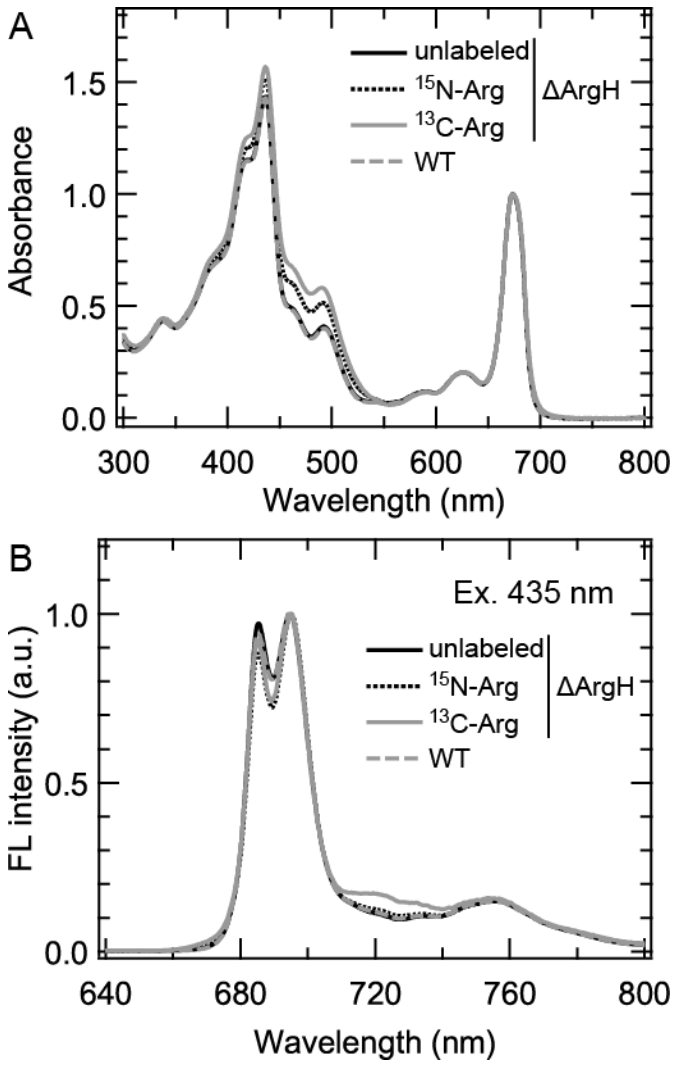

Figure S3: Absorption (A) and fluorescence (B) spectra of isolated PSII complexes from the WT and ΔArgH strains. Absorption and fluorescence spectra were measured at room temperature and −196°C, respectively. The spectra were normalized at the 674-nm and 695-nm peaks in panels A and B, respectively.

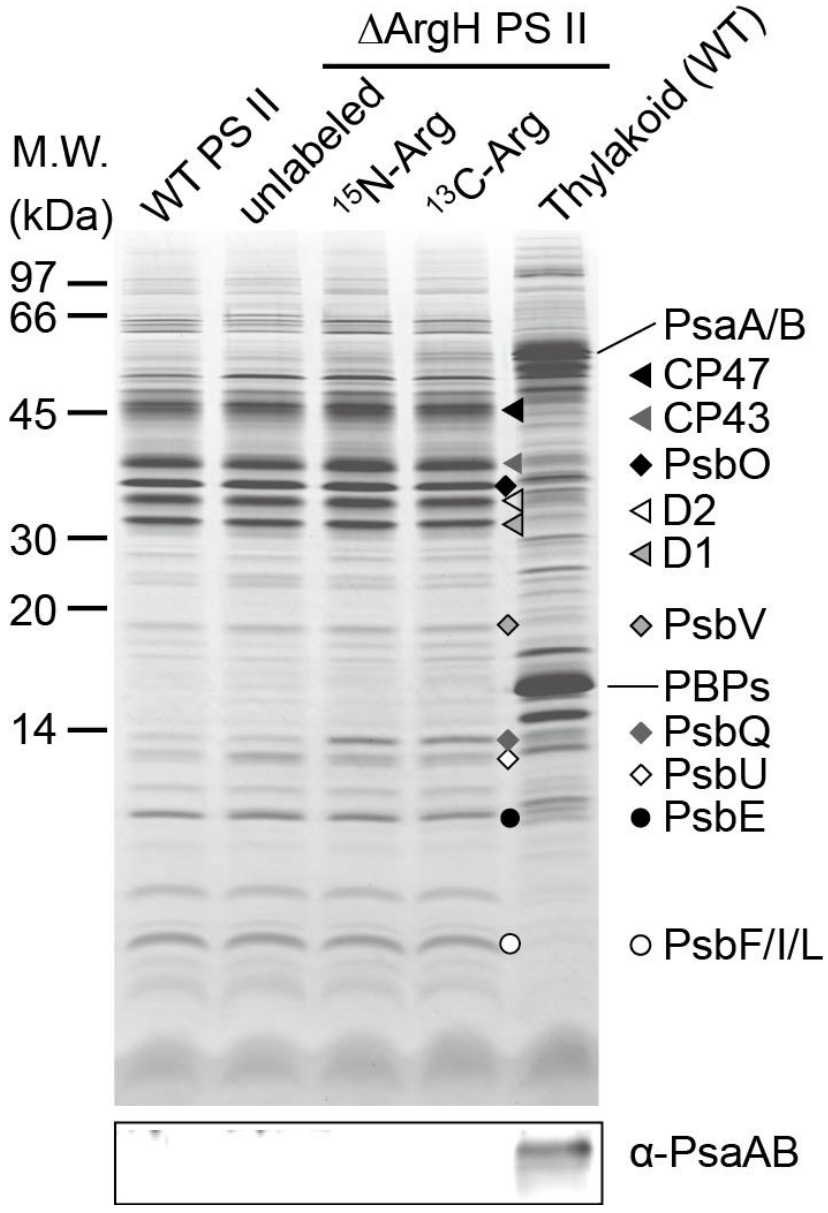

Figure S4: SDS-PAGE analysis of isolated PSII complexes from the ΔArgH strain and Western blotting against the PSI proteins using anti-PsaA/B antibodies (bottom panel).

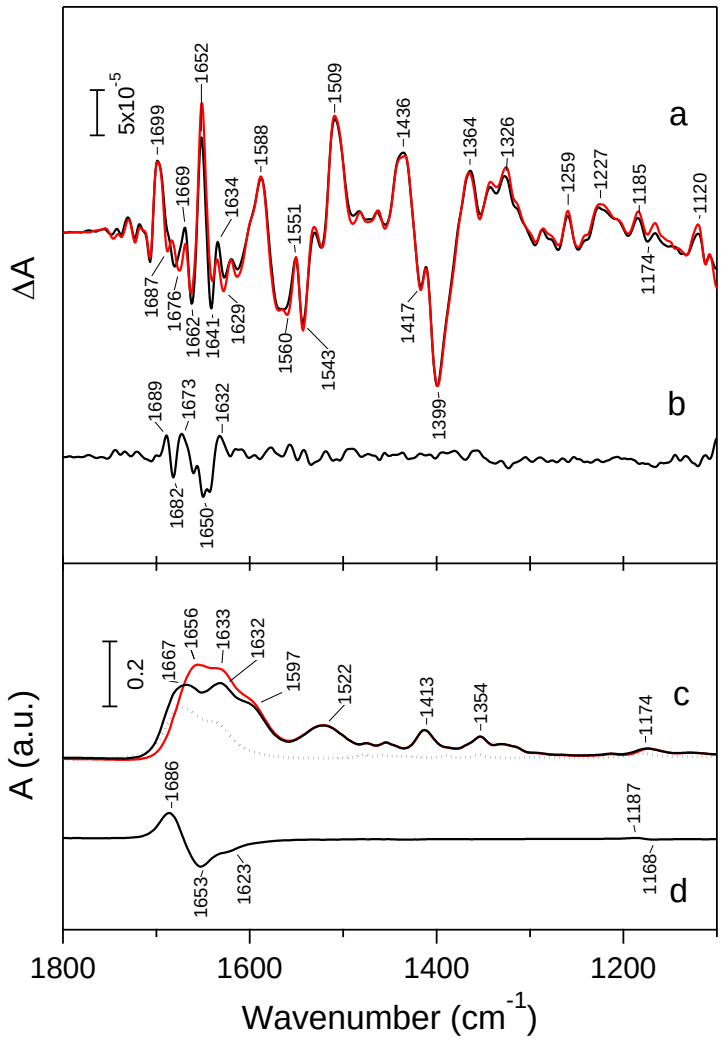

Figure S5: The 1800–1100 cm^{-1} region of the S_2/S_1 FTIR difference spectra of PSII complexes and the FTIR spectra of Arg·HCl and ethylguanidine·HCl in aqueous solutions. (a) S_2/S_1 difference spectra of unlabeled (black line) and [$\eta_{1,2}$-$^{15}N_2$]Arg-labeled (red line) PSII. (b) A double difference S_2/S_1 spectrum between [$\eta_{1,2}$-$^{15}N_2$]Arg-labeled and unlabeled PSII (unlabeled-minus-labeled). (c) FTIR spectra of unlabeled (black solid line) and $\eta_{1,2}$-$^{15}N_2$-labeled (red line) Arg·HCl, and ethylguanidine·HCl (black dotted line) in aqueous solutions. (d) A difference spectra between $\eta_{1,2}$-$^{15}N_2$-labeled and unlabeled Arg·HCl (unlabeled-minus-labeled).

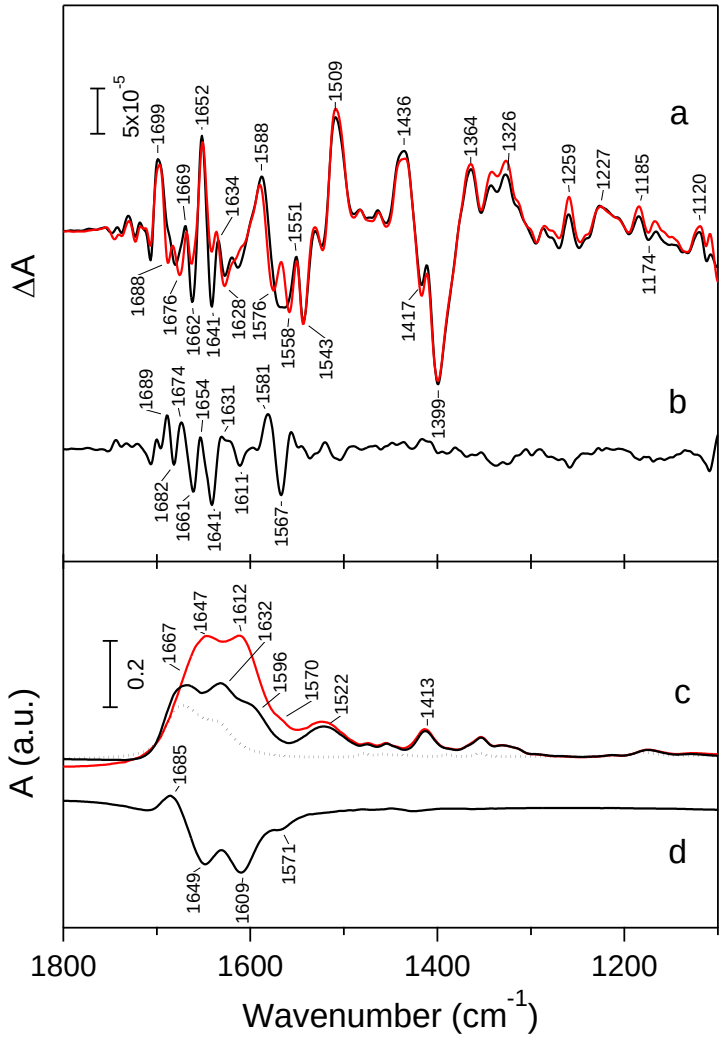

Figure S6: The 1800–1100 cm^{-1} region of the S_2/S_1 FTIR difference spectra of PSII complexes and the FTIR spectra of Arg·HCl and ethylguanidine·HCl in aqueous solutions. (a) S_2/S_1 difference spectra of unlabeled (black line) and [ζ-^{13}C]Arg-labeled (red line) PSII. (b) A double difference S_2/S_1 spectrum between [ζ-^{13}C]Arg-labeled and unlabeled PSII (unlabeled-minus-labeled). (c) FTIR spectra of unlabeled (black solid line) and ζ-^{13}C -labeled (red line) Arg·HCl, and ethylguanidine·HCl (black dotted line) in aqueous solutions. (d) A difference spectra between ζ-^{13}C-labeled and unlabeled Arg·HCl (unlabeled-minus-labeled).