Genetically introduced hydrogen bond interactions reveal an asymmetric charge distribution on the radical cation of the special-pair chlorophyll P680

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The special-pair chlorophyll (Chl) P680 in photosystem II has an extremely high redox potential \( (E_{\text{m}}) \) to enable water oxidation in photosynthesis. Significant positive-charge localization on one of the Chl constituents, \( P_{\text{D1}} \) or \( P_{\text{D2}} \), in P680\(^+\) has been proposed to contribute to this high \( E_{\text{m}} \). To identify the Chl molecule on which the charge is mainly localized, we genetically introduced a hydrogen bond to the 13\(^1\)-keto C=O group on which the charge is mainly localized, we genetically introduced a hydrogen bond to the 13\(^1\)-keto C=O group of \( P_{\text{D1}} \) and \( P_{\text{D2}} \) by changing the nearby D1-Val-157 and D2-Val-156 residues to His, respectively. Successful hydrogen bond formation at \( P_{\text{D1}} \) and \( P_{\text{D2}} \) in the obtained D1-V157H and D2-V156H mutants, respectively, was monitored by detecting 13\(^1\)-keto C=O vibrations in Fourier transfer infrared (FTIR) difference spectra upon oxidation of P680 and the symmetrically located redox-active tyrosines \( Y_Z \) and \( Y_D \), and they were simulated by quantum-chemical calculations. Analysis of the P680\(^+\)/P680 FTIR difference spectra of D1-V157H and D2-V156H showed that upon P680\(^+\) formation, the 13\(^1\)-keto C=O frequency upshifts by a much larger extent in \( P_{\text{D1}} \) (23 cm\(^{-1}\)) than in \( P_{\text{D2}} \) (<9 cm\(^{-1}\)). In addition, thermoluminescence measurements revealed that the D1-V157H mutation increased the \( E_{\text{m}} \) of P680 to a larger extent than did the D2-V156H mutation. These results, together with the previous results for the mutants of the His ligands of \( P_{\text{D1}} \) and \( P_{\text{D2}} \), lead to a definite conclusion that a charge is mainly localized to \( P_{\text{D1}} \) in P680\(^+\).

Photosynthetic water oxidation performed by plants and cyanobacteria produces virtually all oxygen in the atmosphere. This reaction takes place in photosystem II (PSII)\(^3\) protein complexes, which have the function of abstracting electrons from water using light energy releasing protons and molecular oxygen (1–5). The obtained electrons and protons are used to produce NADPH and ATP, respectively, which are utilized to synthesize sugars from CO\(_2\). Photochemistry in PSII starts with light-induced charge separation from the excited singlet state of monomeric chlorophyll (Chl), Chl\(_{\text{D1}}\), coupled with that of the special-pair Chl, P680, to form a radical pair between P680 and a pheophytin (Pheo) electron acceptor, P680\(^+\)Pheo\(^-\) (6, 7). The electron is transferred from Pheo to the primary quinone electron acceptor \( Q_A \) and then the secondary quinone acceptor \( Q_B \), which becomes quinol upon two-electron reduction and is released into thylakoid membranes (8). On the electron donor side, the cation radical of P680 oxidizes the redox-active tyrosine \( Y_Z \) and then the Mn\(_4\)CaO\(_5\) cluster, where two water molecules are oxidized upon four electron transfer reactions (1–5).

P680 has a dimeric structure of two Chl molecules, \( P_{\text{D1}} \) and \( P_{\text{D2}} \), that are bound to the D1 and D2 subunits, respectively (Fig. 1). To achieve water oxidation, which has a redox potential \( (E_{\text{m}}) \) of 880 mV at pH 6.0, P680 needs to be a very strong oxidant. Indeed, its \( E_{\text{m}} \) has been estimated to be ~1200 mV (9–12), which is much higher than the \( E_{\text{m}} \) values of the special pairs of other reaction centers such as P700 of photosystem I (~500 mV (13)) and P870 of bacterial reaction centers (~500 mV (14)). The mechanism that P680 has a high \( E_{\text{m}} \) value has been extensively argued. It was suggested that the high \( E_{\text{m}} \) originates from electrostatic interactions with other cofactors and proteins (15–17), a low dielectric environment (18), and localization of a positive charge on one Chl in the dimer (19, 20).

As for the charge localization on the dimer, electron spin resonance (21) and Fourier transform infrared (FTIR) spectroscopy (22) showed that a spin density or a positive charge on P680 is mostly localized on one Chl in the dimer. These measurements, however, could not specify the Chl molecule, \( P_{\text{D1}} \) or \( P_{\text{D2}} \), on which a charge is mainly distributed. Diner et al. (23) attempted to answer this question by analyzing site-directed mutants of Synechocystis sp. PCC 6803, in which His ligands of \( P_{\text{D1}} \) and \( P_{\text{D2}} \), D1-His-198 and D2-His-197, respectively (24–27), were replaced with other amino acids. They examined the effects of mutations by detecting changes in the visible absorption bands of P680 in difference spectra. Blue shifts of a bleaching band of P680 at 433 nm in the Soret region by up to 3 nm were observed in D1-His-198 mutants, whereas red shifts by 0.5–1.5 nm were observed in D2-His-197 mutants. In the \( Q_y \) region, the bleaching peak at 672.5 nm was blue-shifted by 3 nm upon D1-H198Q mutation. From these results, they proposed...
that the cation is stabilized primarily on P_{D1} (23). A similar Soret shift by D1-H198Q mutation was recently confirmed using *Thermosynechococcus elongatus* (28). Theoretical calculations by Saito et al. (17) and Narzi et al. (29) based on the atomic coordinates of the X-ray crystallographic structure of PSII supported the above view and predicted the asymmetric distribution of a positive charge on P680$^+$ favoring P_{D1} due to the lower $E_m$ of P_{D1} than the $E_m$ of P_{D2}.

Although the above conclusion of charge localization on P_{D1} seems consistent and reasonable, interpretation of the mutation-induced perturbation of the Soret and Q$_o$ bands needs caution. It is known that the excited states of Chls and Pheos in a PSII reaction center are coupled with each other, and the absorption bands of these chromophores are considerably congested in the narrow range of each absorption region. Theoretical works by Renger and co-workers (30–32) showed that the Q$_o$ transition of P680 is formed by the excitonic coupling of P_{D1} and P_{D2} with similar contributions. Indeed, although the experimental absorption difference spectrum in the Q$_y$ region upon P680$^+$ formation was best simulated when the positive charge is localized on P_{D1}, the position of the bleaching peak of P680 was very similar even when a charge was assumed to be localized on P_{D2} (31). The strength of the excitonic coupling between P_{D1} and P_{D2} in the Soret transition has not yet been clarified, and hence the reason for the opposite shifts by mutations of the His ligands of P_{D1} and P_{D2} is not straightforward. Thus, the mutational effects on the changes in the Q$_o$ and Soret bands have not been fully explained to draw a definite conclusion for charge localization on P_{D1} in P680$^+$. A similar Soret shift by D1-H198Q mutation was recently confirmed using *Thermosynechococcus elongatus* (28). Theoretical calculations by Saito et al. (17) and Narzi et al. (29) based on the atomic coordinates of the X-ray crystallographic structure of PSII supported the above view and predicted the asymmetric distribution of a positive charge on P680$^+$ favoring P_{D1} due to the lower $E_m$ of P_{D1} than the $E_m$ of P_{D2}.

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Although the above conclusion of charge localization on P_{D1} seems consistent and reasonable, interpretation of the mutation-induced perturbation of the Soret and Q$_o$ bands needs caution. It is known that the excited states of Chls and Pheos in a PSII reaction center are coupled with each other, and the absorption bands of these chromophores are considerably congested in the narrow range of each absorption region. Theoretical works by Renger and co-workers (30–32) showed that the Q$_o$ transition of P680 is formed by the excitonic coupling of P_{D1} and P_{D2} with similar contributions. Indeed, although the experimental absorption difference spectrum in the Q$_y$ region upon P680$^+$ formation was best simulated when the positive charge is localized on P_{D1}, the position of the bleaching peak of P680 was very similar even when a charge was assumed to be localized on P_{D2} (31). The strength of the excitonic coupling between P_{D1} and P_{D2} in the Soret transition has not yet been clarified, and hence the reason for the opposite shifts by mutations of the His ligands of P_{D1} and P_{D2} is not straightforward. Thus, the mutational effects on the changes in the Q$_o$ and Soret bands have not been fully explained to draw a definite conclusion for charge localization on P_{D1} in P680$^+$. Another way to perturb the P680 property by mutation is changing the hydrogen bond interactions at the 13$^1$-keto C=O groups, which are involved in the macrocycle conjugation of Chls. The change in the hydrogen bond of the 13$^1$-keto C=O can be monitored by FTIR difference or resonance Raman spectroscopy. Indeed, for bacterial reaction centers, various site-directed mutants changing the hydrogen bonds of the 13$^1$-keto C=O groups of bacteriochlorophylls in special pairs have been analyzed using such a spectroscopic method as well as redox potential measurement (14, 33–35), showing that hydrogen bond formation at a 13$^1$-keto C=O group increases the redox potential of a special pair. The merits of vibrational detection of the 13$^1$-keto C=O interaction are as follows. (i) (B)Chl C=O vibrations of individual (B)Chls in a dimer are not coupled with each other and hence can be examined independently. (ii) The 13$^1$-keto C=O vibration is sensitive to a hydrogen bond interaction, and its frequency spans in a wide range depending on the strength of interaction from ~1710 cm$^{-1}$ for free interaction to ~1650 cm$^{-1}$ for strong hydrogen bonding (36–38). (iii) Upon cation formation, the 13$^1$-keto C=O frequency upshifts by ~30 cm$^{-1}$ in monomeric (B)Chl (39–41), and the extent of the shift reflects the charge distribution on each (B)Chl molecule in a dimer (22, 34). Thus, using vibrational spectroscopy, the genetically perturbed hydrogen bond of the 13$^1$-keto C=O of each (B)Chl molecule in a dimer can be independently monitored, and the charge distribution can be examined.

Light-induced FTIR difference spectroscopy has been extensively used to investigate the structural changes and photochemical reactions in PSII at a molecular level (42–48). As for P680, light-induced FTIR difference spectra upon photo-oxidation of P680 have been measured using various PSII preparations (22, 49–51). It has been shown that a single negative band due to neutral P680 was detected at ~1700 cm$^{-1}$, representing a free interaction of the 13$^1$-keto C=O group. This is consistent with the X-ray crystal structure of PSII core complexes (24–27), where amino acid residues neighboring the 13$^1$-keto C=O bonds of P_{D1} and P_{D2} are non-hydrogen-bonding D1-Val-157 and D2-Val-156, respectively (Fig. 1). For P680$^+$, two positive peaks were observed at ~1724 and ~1710 cm$^{-1}$ in PSII membranes and core complexes (22, 50, 51). From the upshifts by ~24 and ~10 cm$^{-1}$ from the neutral P680 band, it was proposed that more than 70% of a charge is distributed on one Chl in P680$^+$ (22). To identify the Chl molecule that mainly possesses a positive charge on P680$^+$, the assignment of these two bands to either P_{D1} or P_{D2} is required. Such an assignment can be achieved by introduction of a hydrogen bond to the 13$^1$-keto C=O of P_{D1} and P_{D2}.
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question for the assignments of these signals. If these assignments are confirmed, the differential signals around 1700 cm\(^{-1}\) in the \(Y_Z/Y_D\) and \(Y_D\) spectra can be useful markers for monitoring the 13\(^1\)-keto C=O interactions of P\(_{D1}\) and P\(_{D2}\).

In this study, we mutated D1-Val-157 and D2-Val-156 to His to introduce a hydrogen bond at the 13\(^1\)-keto C=O group of P\(_{D1}\) and P\(_{D2}\), respectively, using a cyanobacterium Synechocystis sp. PCC 6803 (Fig. 1). The obtained D1-V157H and D2-V156H mutants were analyzed by detecting light-induced FTIR spectra of P680, Y\(_Z\), and Y\(_D\) upon their light-induced oxidation. The interactions of the 13\(^1\)-keto C=O groups in these mutants and the frequency changes were also simulated by quantum mechanics/molecular mechanics (QM/MM) calculations based on the high-resolution X-ray crystallographic structure (26). Furthermore, the effect of mutations on the redox potential of P680 was examined using thermoluminescence (TL) measurements. The obtained data showed successful introduction of hydrogen bonds to the 13\(^1\)-keto C=O groups of P\(_{D1}\) and P\(_{D2}\) and provided solid evidence for the main distribution of a positive charge on P\(_{D1}\) in P680\(^{+}\).

Results

Cell growth and \(O_2\) evolution activity of the D1-V157H and D2-V156H mutants

In this study, we used four strains of Synechocystis sp. PCC 6803 as follows: D1-WT, D2-WT, D1-V157H, and D2-V156H. In D1-WT, the psbA1 and psbA3 genes were deleted, and PsbA2 was expressed as a D1 protein; in D2-WT, the psbD2 gene was deleted, and PsbD1 was expressed as a D2 protein. These PsbA2 and PsbD1 proteins in D1-WT and D2-WT, respectively, have native sequences, and thus they were designated D1- and D2-“WT”. In contrast, in the D1-V157H and D2-V156H mutants, D1-Val-157 and D2-Val-156, respectively, were replaced with His. Both of D1-V157H and D2-V156H as well as D1-WT and D2-WT grew photoautotrophically.

The \(O_2\) evolution activities of D1-V157H and D2-V156H were 400–440 and 440–490 μmol of \(O_2\) (mg of Chl)\(^{-1}\) h\(^{-1}\), respectively, although those of corresponding wild-type species (D1-WT and D2-WT, respectively) were 550–610 and 540–620 μmol of \(O_2\) (mg of Chl)\(^{-1}\) h\(^{-1}\).

Thermoluminescence

TL measurements were performed using cells of the four strains in the presence of DCMU. The TL glow curves obtained after illumination at \(-20^\circ C\) provided the so-called Q band (59), which originates from S\(_2\)Q\(_A^-\) recombination (Fig. 2). The features of the glow curves of the D1-WT (Fig. 2a, black line) and D2-WT (b, black line) cells were virtually identical, showing a Q band at 11 °C. In contrast, the D1-V157H mutation (Fig. 2a, red line) upshifted the Q band by 17 °C to give a peak at 28 °C with an increased intensity by a factor of 2.05, whereas D2-V156H mutation (b, blue line) induced only a small upshift by 2 °C showing a peak at 14 °C with a slight intensity increase by a factor of 1.22 (Table 1).

P680\(^{+}\)/P680 FTIR difference spectra

Fig. 3A shows light-induced P680\(^{+}\)/P680 FTIR difference spectra (1800–1100 cm\(^{-1}\)) of the PSII core complexes from the D1-V157H (trace a, red line) and D2-V156H (trace b, blue line) mutants in comparison with the spectra of D1-WT (trace a, black line) and D2-WT (trace b, black line), respectively. Positive and negative bands represent the cationic and neutral forms of P680, respectively. The spectra of D1-WT and D2-WT were virtually identical, indicating that there is no effect of deletion of the psbA1 and psbA3 genes in D1-WT and of the psbD2 gene in D2-WT on the P680 interaction. These WT spectra are very similar to the previously reported P680\(^{+}\)/P680 FTIR difference spectra of cyanobacterial PSII core complexes and spinach PSII membranes (22, 50, 51). Prominent bands in the 1730–1800 cm\(^{-1}\) region have been attributed to the chlorin ring vibrations (22, 36–41, 49–51). In addition, prominent bands at 1690–1700 cm\(^{-1}\) have been attributed to its chlorin ring vibrations (22, 36–41, 49–51). In addition, prominent bands at 1690–1700 cm\(^{-1}\) have been assigned to the 13\(^1\)-keto C=O stretching vibrations, respectively, of P680, although medium intensity bands at 1620–1600 cm\(^{-1}\) have been attributed to its chlorin ring vibrations (22, 36–41, 49–51). In addition, prominent bands at 1690–1620 cm\(^{-1}\) can be assigned to the amide I vibrations due to the CO stretches of polypeptide main chains upon cation formation on P680. The overall features of the P680\(^{+}\)/P680 spectra of the D1-V157H (Fig. 3A, trace a, red line) and D2-V156H (Fig. 3A, trace b, blue line) mutants were very similar to those of the WT species (trace a and b, black lines). In particular, bands of the chlorin ring vibra-
tions (1620–1100 cm\(^{-1}\)) were virtually unaffected by mutations. In contrast, some specific changes were observed in the 131-keto C=O region (1730–1690 cm\(^{-1}\)).

The expanded view of the 131-keto C=O region of the P680\(^{1+}\) spectra is shown in Fig. 3B. In the WT spectra (Fig. 3B, traces a and b, black lines), a prominent negative band at 1697 cm\(^{-1}\) and two positive bands at 1726 and 1708 cm\(^{-1}\) have been assigned to the 131-keto C=O vibrations of neutral and cationic forms of P680, respectively (22). The single band at 1697 cm\(^{-1}\) was interpreted as the overlap of the two C=O bands from P\(_{D1}\) and P\(_{D2}\), both of which are free from hydrogen bond interactions. In contrast, the split bands at 1726 and 1708 cm\(^{-1}\) of P680\(^{1+}\) were attributed to the asymmetric distribution of a positive charge on P\(_{D1}\) and P\(_{D2}\) (22). The D1-V157H spectrum (Fig. 3B, trace a, red line) also exhibited two positive bands at 1726 and 1708 cm\(^{-1}\) but changed their relative intensities. The 1726-cm\(^{-1}\) band was weakened, whereas that at 1708 cm\(^{-1}\) was strengthened. In addition, there was an intensity increase around 1720 cm\(^{-1}\) most likely due to the appearance of a new band. In neutral P680, the negative band at 1697 cm\(^{-1}\) slightly downshifted to 1695 cm\(^{-1}\) with a weakened intensity.

The D2-V156H spectrum (Fig. 3B, trace b, blue line) showed a much clearer change. The negative band at 1697 cm\(^{-1}\) of WT disappeared, leaving a half-intensity band at 1703 cm\(^{-1}\). Instead, a new band appeared at 1680 cm\(^{-1}\) superimposing an original band at 1682 cm\(^{-1}\). This change is best interpreted as that one of the bands that contribute to the intensity at 1697 cm\(^{-1}\) downshifted by 17 cm\(^{-1}\) to 1680 cm\(^{-1}\), whereas another band was left at 1703 cm\(^{-1}\). Along with this change, a positive shoulder at 1708 cm\(^{-1}\) seemed to downshift by 19 cm\(^{-1}\) to 1689 cm\(^{-1}\). In contrast, the main positive band at 1726 cm\(^{-1}\) was virtually unchanged upon the D2-V156H mutation.

YZ/\(\gamma_z\) and Y\(_D^{+}\)/\(\gamma_d\) FTIR difference spectra

Fig. 4A shows light-induced Y\(_Z^{+}\)/\(\gamma_z\) FTIR difference spectra of PSII complexes from D1-WT (a, black line), D1-V157H (a, red line), D2-WT (b, black line), and D2-V156H (b, blue line) in the region of 1800–1200 cm\(^{-1}\). Overall spectral features were very similar between mutants and WT species. In particular, a positive band at 1512 cm\(^{-1}\) and a negative band at 1257 cm\(^{-1}\), which have been assigned to the CO stretching vibration of oxidized Y\(_Z^{+}\) and the coupled mode of the CO stretching and COH bending vibrations of reduced Y\(_Z\), respectively (55, 57, 58), were virtually unaffected by mutations. In contrast, some specific changes were observed in the 131-keto C=O region (1730–1690 cm\(^{-1}\)).
showed similar characteristics, although these peaks slightly downshifted in D1-V157H, indicative of minor perturbation of the YZ hydrogen bond in this mutant. An expanded view of the YZ/H18528/YZ spectra in the region of the 131-keto C/H11005/O bands of P680 (Fig. 4B) showed a clear change in the D1-V157H spectrum. The 1707/1699-cm\(^{-1}\) peaks in D1-WT (Fig. 4B, a, black line) downshifted to 1703/1697 cm\(^{-1}\) by 2–4 cm\(^{-1}\) in D1-V157H (Fig. 4B, a, red line), concomitantly with some intensity decrease. In contrast, there was no change in these peaks in the D2-V156H spectrum (Fig. 4B, b, blue line).

Fig. 5A shows light-induced YD’/YD FTIR difference spectra of the PSII core complexes in the 1800–1200-cm\(^{-1}\) region (A) and the expanded view of the 131-keto C=O stretching region of P680 (B). a, D1-WT (black) and D1-V157H (red), b, D2-WT (black) and D2-V156H (blue). The spectra were normalized by the intensity of the CO stretching band of the YD’ radical at 1503 cm\(^{-1}\).

Figure 5. YD’/YD FTIR difference spectra of the PSII core complexes in the 1800–1200-cm\(^{-1}\) region (A) and the expanded view of the 131-keto C=O stretching region of P680 (B). a, D1-WT (black) and D1-V157H (red), b, D2-WT (black) and D2-V156H (blue). The spectra were normalized by the intensity of the CO stretching band of the YD’ radical at 1503 cm\(^{-1}\).

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Fig. 5A shows light-induced YD’/YD FTIR difference spectra of PSII complexes from D1-WT (a, black line), D1-V157H (a, red line), D2-WT (b, black line), and D2-V156H (b, blue line) in the 1800–1200-cm\(^{-1}\) region. Overall spectral features were very similar among the spectra, including the CO band of oxidized YD’ at 1503 cm\(^{-1}\) and the CO/COH band of reduced YD at 1251 cm\(^{-1}\) (52, 53, 61). However, a considerable change was observed in the 131-keto C=O region of P680 in the D2-V156H spectrum (Fig. 5B, b). The 1702/1695-cm\(^{-1}\) peaks in D2-WT (Fig. 5B, b, black line) completely disappeared, and instead a large positive peak appeared at 1676 cm\(^{-1}\). A corresponding negative peak may be a dip at 1684 cm\(^{-1}\), whose intensity is relatively small probably because an original positive peak at 1688 cm\(^{-1}\) overlaps. This spectral change indicates that the 1702/1695-cm\(^{-1}\) peaks downshifted by 18–19 cm\(^{-1}\) upon the D2-V156H mutation. In sharp contrast, the corresponding peaks at 1703/1695 cm\(^{-1}\) in D1-WT (Fig. 5B, a, red line) were unchanged upon the D1-V157H mutation (Fig. 5B, a, red line).

QM/MM calculations of the 131-keto C=O frequencies of P680 models

To rationalize the observed changes in the 131-keto C=O frequencies of P680 by mutations, we performed QM/MM calculations of the P680 models of WT and the mutants. The QM region of WT consists of PD1, PD2, D1-His-198, D2-His-197, D1-Val-157, and D2-Val-156) are expressed as balls, and cofactors, amino acid residues, and water molecules in the MM region are shown as tubes (cofactors) and wire frame (others) images. The phytol chains of PD1 and PD2 are colored green, and the monomeric Chls (ChlD1 and ChlD2) and Pheos (PheoD1 and PheoD2) are colored blue and magenta, respectively.

Figure 6. QM and MM regions of P680 model (WT) used for QM/MM calculations (A, top view; B, side view). Atoms in the QM region (the headgroups of PD1 and PD2, and the side chains of D1-His-198, D2-His-197, D1-Val-157, and D2-Val-156) are expressed as balls, and cofactors, amino acid residues, and water molecules within 10 Å from PD1 and PD2 were assigned to the MM region (Fig. 6). For the models of the D1-V157H and D2-V156H mutants, the side chains of D1-Val-157 and D2-Val-156, respectively, were replaced with a His side chain. The optimized geometries of the QM region of these P680 models are shown in Fig. 7, and the calculated 131-keto C=O frequencies are presented in Table 2 together with the values of the hydrogen bond distances and angles. In both of the mutant models,
the His side chain was hydrogen-bonded to the 131'-keto C=O oxygen at the imidazole Nε-H (proximal NH). The structure that has a hydrogen bond at the Nε-H (distal NH) was not converged in the D1-V157H model, and it provided a less stable form than the Nε-H hydrogen-bonded form with a higher energy by 10.8 kcal/mol in the D2-V156H model.

In the WT model, the 131'-keto C=O frequency of PD2 was calculated to be lower by 9 cm⁻¹ than that of PD1 (1692 cm⁻¹ versus 1703 cm⁻¹), representing an asymmetric P680 structure. In the D1-V157H model, the His NH formed a rather weak hydrogen bond with the 131'-keto C=O of PD1 with a hydrogen bond distance (H−O) of 2.61 Å and an angle (N−H−O) of 96.4° (Table 2). Indeed, only a small downshift by 4 cm⁻¹ of the 131'-keto C=O frequency of PD1 was estimated. In contrast, the D2-V156H model formed a relatively strong hydrogen bond between the His and the 131'-keto C=O of PD2 with a distance (H−O) of 1.94 Å and an angle of 124.8°, resulting in a large downshift of the 131'-keto C=O frequency by 17 cm⁻¹ (Table 2). It is notable that in these mutant models, the 131'-keto C=O frequency of the other side of Chl without mutation (i.e. PD2 in D1-V157H and PD1 in D2-V156H) was unaffected, indicative of the independence of the 131'-keto C=O vibrations of PD1 and PD2.

**Discussion**

In this study, we identified the Chl molecule (PD1 or PD2) in which a positive charge is mainly localized on P680⁺ by analyzing the site-directed mutants, D1-V157H and D2-V156H, of *Synechocystis* sp. PCC 6803, which are expected to introduce a hydrogen bond interaction to the 131'-keto C=O group of PD1 and PD2, respectively (Fig. 1). Both mutants showed clear changes in TL glow curves (Fig. 2) and the 131'-keto C=O stretching region of the P680⁺/P680 FTIR difference spectra (Fig. 3), indicating successful hydrogen bond formation to P680. In addition, the D1-V157H mutation induced a downshift of the 1707/1699 cm⁻¹ differential signal in the YZ/YD FTIR difference spectrum (Fig. 4, trace a) by 4 cm⁻¹ without any change in the YD/YD spectrum (Fig. 5, trace a), whereas D2-V156H mutation induced a downshift of the similar differential signal at 1703/1695 cm⁻¹ in the YD/YD spectrum by 19 cm⁻¹ (Fig. 5, trace b) without a change in the YZ/YZ spectrum. These observations in the YZ/YZ and YD/YD spectra confirmed the hydrogen bond formation at PD1 in D1-V157H and at PD2 in D2-V156H. In addition, the observations provided definite assignments of the differential signals at ~1700 cm⁻¹ in the YZ/YZ and YD/YD spectra to the 131'-keto C=O vibrations of PD1 and PD2, respectively, excluding the possibility of the assignments to the peptide C=O vibrations (53–55).

It has been shown that upon oxidation of YZ, its proton is transferred to the neighboring D1-His-190 that becomes a protonated cation (57, 62–64), whereas a proton from YD is released to the bulk (58, 65), and hence a positive charge is not accumulated around YD. Thus, the similar band shifts of the 131'-keto C=O of PD1 and PD2 by YZ and YD formation, respectively, may be caused by a common change in the protein environment around the C=O group induced by tyrosine radical formation rather than an electrochromic effect. In any case, it is now clear that the differential signals at ~1700 cm⁻¹ in the YZ/YZ and YD/YD spectra are useful markers to independently monitor the 131'-keto C=O vibrations of PD1 and PD2, respectively, in neutral P680 without interference of the bands of P680⁺. Because in both cases the frequency difference between the two peaks of the differential signal (8 cm⁻¹) is similar to the width of a 131'-keto C=O band (for example, the full width of half-maximum of the 131'-keto C=O band at 1726 cm⁻¹ is

**Table 2**

|                | P_D1 | P_D2   |
|----------------|------|--------|
| rC=O cm⁻¹     | Δν cm⁻¹ | r Å  | θ°    |
| WT             | 1703 |        | 1692  | 1692  |
| D1-V157H      | 1699 | -4     | 2.61  | 96.4  |
| D2-V156H      | 1703 | 0      | 1675  | 17     |

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* C=O frequencies were scaled with a scaling factor of 0.9416 to adjust the calculated frequency of P_D1 in the WT model to the experimental frequency, 1703 cm⁻¹.
* Frequency shift from WT is shown.
* Hydrogen bond distance between the oxygen atom of 131'-keto C=O and the hydrogen atom of the Nε-H of His is shown.
* Hydrogen bond angle is shown.

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**Asymmetric charge distribution on P680⁺ in PSII**
Asymmetric charge distribution on P680\(^+\) in PSII

| Table 3 |

| Species | \(P_{D1}/P_{D2}\) | \(P680\) | \(P680^+\) | FTIR spectra |
| --- | --- | --- | --- | --- |
| WT | \(P_{D1}\) | 1703\(^a\) | 1726 | \(Y_2'/Y_1'\); \(P680^+\)/P680 |
|  | \(P_{D2}\) | >1697\(^b\) | <1708\(^a\) | \(Y_2'/Y_1'\); P680/P680 |
| D1-V157H | \(P_{D1}\) | 1700\(^c\) (<3\(^c\)) | 1726, ~1720 (~6\(^c\)) | \(Y_2'/Y_1'\); P680/P680 |
|  | \(P_{D2}\) | >1695\(^b\) | <1708\(^a\) | \(Y_2'/Y_1'\); P680/P680 |
| D2-V156H | \(P_{D1}\) | 1703\(^a\) | 1726 | \(Y_2'/Y_1'\); \(P680^+\)/P680 |
|  | \(P_{D2}\) | 1680\(^c\) (<19\(^c\)) | ~1689 (~19\(^c\)) | \(Y_2'/Y_1'\); \(P680^+\)/P680 |

\(^a\) A middle frequency of the two peaks in a differential signal is shown.

\(^b\) An apparent peak frequency is shown, which is slightly shifted from the true frequency by a band overlap.

\(^c\) A shift from the WT value is given in parentheses.

about 10 \(cm^{-1}\), it is conceivable that this differential shape arises from a small shift of the original band. Hence, the position of the original band can be roughly estimated as a middle point of the two peak frequencies. Thus, from the 1707/1699- and 1703/1695 \(cm^{-1}\) peaks in the \(Y_2'/Y_1'\) and \(Y_{1D}/Y_{1D}\) spectra of WT (Figs. 4 and 5), the \(131\)-keto C=O frequencies of \(P_{D1}\) and \(P_{D2}\) in neutral P680 are estimated to be 1703 and 1699 \(cm^{-1}\), respectively (Table 3). The lower \(P_{D2}\) frequency by 4 \(cm^{-1}\) than \(P_{D1}\) may reflect an asymmetric P680 structure or an asymmetric protein environment around the \(131\)-keto C=O groups.

Upon D1-V157H mutation, the differential signal around 1703 (1707/1699) \(cm^{-1}\) in the \(Y_{2D}/Y_{1D}\) difference spectrum downshifted by ~3 \(cm^{-1}\) to that around 1700 (1703/1697) \(cm^{-1}\) (Fig. 4B, trace a; Table 3). In contrast, the D2-V156H mutation induced a large downshift of the differential signal by ~19 \(cm^{-1}\) from 1699 (1702/1695) to 1680 (1684/1676) \(cm^{-1}\) (Fig. 5B, trace b; Table 3). Thus, \(P_{D2}\) forms a relatively strong hydrogen bond with the introduced His in the D2-V156H mutant, whereas \(P_{D1}\) forms only a weak hydrogen bond with the His in D1-V157H. These frequency shifts of the \(131\)-keto C=O vibrations are consistent with the mutation-induced changes in the \(131\)-keto C=O frequencies of PD1 in D1-V157H and PD2 in neutral P680 are estimated to be 1703 and 1699 \(cm^{-1}\), respectively (Table 3). The lower \(P_{D2}\) frequency by 4 \(cm^{-1}\) than \(P_{D1}\) may reflect an asymmetric P680 structure or an asymmetric protein environment around the \(131\)-keto C=O groups.

upon the high-resolution (1.9 Å) X-ray crystal structure of WT (Fig. 7; Table 2) (26). It was found that the space around the \(131\)-keto C=O group of \(P_{D1}\) is too narrow to fit the His side chain, and only a weak hydrogen bond with a long hydrogen bond distance (O–H) of 2.61 Å and a non-proper angle of 96.4° was formed in D1-V157H (Fig. 7B; Table 2). A small downshift of the C=O frequency by 4 \(cm^{-1}\) was calculated, reproducing the experimental downshift of 3 \(cm^{-1}\). In contrast, a His side chain fits better to the space around the \(131\)-keto C=O of \(P_{D2}\). A much stronger hydrogen bond with His was formed in D2-V156H with a shorter hydrogen bond distance of 1.94 Å and a better angle of 124.8° (Fig. 7C; Table 2). It was found that the space around the \(131\)-keto C=O group between \(P_{D1}\) and \(P_{D2}\) is reflected by a shorter distance between the oxygen atom of the \(131\)-keto C=O and the Ce of the Val residue for \(P_{D1}\) (5.3 Å) than that for \(P_{D2}\) (5.8 Å) in the X-ray structure of WT (26). Thus, the asymmetric protein environment of P680 is the primary cause for the difference between the hydrogen bond strengths in the two mutants that introduced a His side chain for Val. In WT, the \(131\)-keto C=O frequency of \(P_{D2}\) was calculated to be lower by 9 \(cm^{-1}\) than that of \(P_{D1}\) (Table 2), reproducing the experimental tendency that showed a lower frequency by 4 \(cm^{-1}\) (Table 3). It was also shown that the C=O frequencies of the non-perturbed Chls in the mutants, i.e. \(P_{D2}\) in D1-V157H and \(P_{D1}\) in D2-V156H, were unchanged, reinforcing the independency of the two \(131\)-keto C=O vibrations of P680.

In monomeric Chl, cation formation generally upshifts the \(131\)-keto C=O frequency by ~30 \(cm^{-1}\) (39–41). In a Chl dimer, the extent of upshift becomes smaller depending on the ratio of charge distribution between two Chls (22, 34). In the P680\(^+\)/P680 difference spectrum of WT (Fig. 3B, black line), two positive bands due to the \(131\)-keto C=O vibrations of P680\(^+\) were observed at 1726 and 1708 \(cm^{-1}\), which were upshifted from a negative band at 1697 \(cm^{-1}\) consisting of two bands at 1703 and 1699 \(cm^{-1}\) (Table 3). The lower frequency of the 1697-\(cm^{-1}\) peak than the frequencies of the two constituents suggests that at least one of the two bands upshifts by a smaller extent than the bandwidth upon P680 oxidation, most probably forming a weak positive band at 1708 \(cm^{-1}\). Hence, the original frequency of the latter band should also be slightly...
lower than 1708 cm\(^{-1}\). The presence of the two bands in P680\(^+\) was previously attributed to the localization of 70–80% of a charge on the Chl showing the higher-frequency band at 1726 cm\(^{-1}\) (22). The assignments of the two 13\(^1\)-keto C=O bands of P680\(^-\) were achieved by inspection of the P680\(^-\)/P680 spectra of the D1-V157H and D2-V156H mutants (Fig. 3). In the D2-V156H mutant, the 1708-cm\(^{-1}\) band downshifted to \(\sim1689\) cm\(^{-1}\) by \(\sim19\) cm\(^{-1}\), the same extent as the shift of the neutral form, whereas the 1726-cm\(^{-1}\) band was unchanged (Fig. 3B, trace b). In contrast, in the D1-V157H mutant, the 1726-cm\(^{-1}\) band decreased the intensity concomitantly with the appearance of a broad feature around 1720 cm\(^{-1}\), indicative of a downshift by \(\sim6\) cm\(^{-1}\) of the 13\(^1\)-keto C=O band in partial centers, whereas the frequency of the 1708-cm\(^{-1}\) band was little affected. The observation of the remaining intensity at 1726 cm\(^{-1}\) and a broad feature of the band around 1720 cm\(^{-1}\) may be attributed to an unstable hydrogen bonding interaction between the introduced His and the P\(_{D1}\) C=O, which could cause destruction of the weak hydrogen bond in some centers. The above observations in the two mutants provide clear assignments of the 1726- and 1708-cm\(^{-1}\) bands between the introduced His and the PD1 C=O, respectively, in P680\(^-\). It is therefore concluded that a positive charge is mainly localized on P\(_{D1}\), providing a larger upshift.

This conclusion is consistent with the mutation effects on the TL glow curves due to S\(_2\)Q\(_{A\_}\) recombination in the presence of DCMU (Fig. 2; Table 1). The D1-V157H mutant largely upshifted the peak temperature from 11 to 28 °C by 17 °C concomitantly with a significant intensity increase by a factor of 2.05 (Fig. 2a). In contrast, the D2-V156H mutant upshifts the peak temperature only by 3 °C with a small intensity increase by a factor of 1.22 (Fig. 2b). It is known that the S\(_2\)Q\(_{A\_}\) recombination takes place mainly through a relaxation pathway via P680\(^-\)Pheo\(^-\) (66, 67), and the quantum yield of emission through P680\(^-\) is very small (3% in WT (68)). Thus, the peak temperature of the TL band mainly reflects \(\Delta G\) between P680\(^-\)Pheo\(^-\) and S\(_2\)Q\(_{A\_}\), whereas the TL intensity reflects \(\Delta G\) between P680\(^-\) and P680\(^-\)Pheo\(^-\) (66, 67). These energy gaps are expressed using the redox potentials of the components shown in Equations 1 and 2,

\[
\Delta G(P680\(^-\)Pheo\(^-\) - S\(_2\)Q\(_{A\_}\)) = \Delta G(P680\(^-\)) - E_m(P680/P680\(^+\)) - E_m(S\(_2\)/S\(_2\)) - E_m(Pheo\(^-\)/Pheo) \quad (\text{Eq. 1})
\]

\[
\Delta G(P680\(^+\) - P680\(^-\)Pheo\(^-\)) = \Delta G(P680\(^+\)) - E_m(P680/P680\(^+\)) - E_m(P680/P680\(^-\)) \quad (\text{Eq. 2})
\]

where \(F\) is the Faraday constant, and \(\Delta G(P680\(^+\)-P680\(^-\))\) is the transition energy of P680 that has a constant value of 1.8 eV. In the D1-V157H and D2-V156H mutants that perturbed the hydrogen-bonding property of P680, \(E_m(P680/P680\(^+\))\) is expected to be mainly affected. Thus, the observed upshifts of the peak temperature, which represent the increases in \(\Delta G(P680\(^-\)Pheo\(^-\) - S\(_2\)Q\(_{A\_}\))\), indicate the upshifts of \(E_m(P680/P680\(^+\))\), to a larger extent in D1-V157H than in D2-V156H. The mutation-induced change in \(\Delta G(P680\(^-\)Pheo\(^-\))\), \(\Delta G(P680\(^+\)-P680\(^-\))\), which corresponds to the change in \(E_m(P680/P680\(^+\))\), \(\Delta G(P680\(^-\))\), according to Equation 2, is expressed using the ratio of the TL intensity of the mutant \((I'/I)\) to that of WT \((I)\) as shown in Equation 3,

\[
\Delta G(P680\(^-\)Pheo\(^-\)) = \Delta G(P680\(^+\)-P680\(^-\)) = -RT_m\ln(I'/I) \quad (\text{Eq. 3})
\]

where \(T_m\) is the peak temperature. Using Equation 3 and the observed intensity increase by mutation, \(\Delta G(P680\(^+\)-P680\(^-\))\) was estimated to be +18.1 ± 0.7 and +4.9 ± 0.4 mV in D1-V157H and D2-V156H, respectively (Table 1), in agreement with the tendency predicted by the shifts of the peak temperature. It is noted that the obtained \(\Delta G(P680\(^+\)-P680\(^-\))\) values are based on a rather simplified assumption in the thermoluminescence theory, and hence they may be considered as approximate estimations. Nevertheless, the larger \(\Delta G(P680\(^+\)-P680\(^-\))\) change by the perturbation of P\(_{D1}\), despite much weaker hydrogen bonding at P\(_{D1}\) than P\(_{D2}\), is consistent with the FTIR conclusion that a positive charge is mainly distributed to P\(_{D1}\) in P680\(^-\). In addition, the 18-mV increase is consistent with the weak hydrogen bond in D1-V157H, taking into account the 60–80-mV increase in \(\Delta G(P680/P680\(^+\))\) by formation of a proper hydrogen bond with His at the 13\(^1\)-keto C=O of bacteriochlorophyll observed in the studies of bacterial reaction centers (14, 34).

The conclusion in this study, i.e., charge localization on P\(_{D1}\) in P680\(^-\), obtained using the mutants introducing hydrogen bonds to P\(_{D1}\) and P\(_{D2}\), is basically consistent with that of the previous study by Diner et al. (23), which used the mutants of the His ligands of these Chls. They analyzed the mutants by detecting the changes in the Soret and Q\(_y\) bands. Although excitonic couplings among the chromophores were not considered in their analysis, later theoretical studies by Renger and co-workers (30–32) showed that the excited states of P\(_{D1}\) and P\(_{D2}\) are appreciably coupled with each other. It was suggested that P\(_{D1}\) and P\(_{D2}\), which have site energies at 666 nm in the Q\(_y\) transitions, form two delocalized exciton states at ~675 and ~658 nm (31). The experimental observation of a blue shift of the Q\(_y\) bleach at 672.5 nm by 3 nm upon D1-H198Q mutation (23) was well simulated by a blue shift of the site energy of P\(_{D1}\) by 8 nm (31). Because the experimental data of the Q\(_y\) shifts by D2-His-197 mutations were not reported (23), the contribution of P\(_{D2}\) to the bleaching band was not revealed experimentally. In the Soret region, Diner et al. (23) observed blue shifts by up to 3 nm upon D1-His-198 mutations and red shifts by 0.5–1.5 nm upon D2-His-197 mutations. This opposite displacement in the D2-His-197 mutants was explained by the position of the P\(_{D2}\) band at a longer wavelength (436 nm) than that of D\(_{D1}\) band (433 nm) and the increased contribution of the P\(_{D2}\) band to the difference spectrum due to the change in \(E_m(P680\(^-\))\). However, the excitonic couplings of the Soret transitions have not been theoretically clarified, and hence the assumption of a weak coupling between P\(_{D1}\) and P\(_{D2}\) was not guaranteed. In contrast, in our study, the 13\(^1\)-keto C=O vibrations of P\(_{D1}\) and P\(_{D2}\) which were detected by FTIR spectroscopy, are independent of each other, and a genetically introduced hydrogen bond changed only the C=O frequency of the perturbed Chl (Table 3). Therefore, our results for the perturbations of the 13\(^1\)-keto C=O
Asymmetric charge distribution on P680⁺ in PSII

vibrations, together with the results for the perturbations of the electronic transitions by Diner et al. (23), lead to a definite conclusion that a positive charge mainly resides on P₃₁.

Localization of a positive charge on P₃₁ is crucial in the physiological function of P680. A density functional theory (DFT) calculation of a P680 model previously estimated that charge localization on one Cyt b₅₅₉, where the electronic state of P680⁺ is significantly altered and a positive charge is rather delocalized over P₃₁ and P₃₂ (22), P680⁺ does not have a function of Y₂ oxidation anymore. In addition to providing a driving force of Y₂ oxidation by the high Eₘₚ value, the presence of a charge on P₃₁ may be advantageous to the electron transfer rate from Y₂ to P680⁺ due to the closer distance from Y₂ to P₃₁ than to P₃₂ (the edge-to-edge distance of the conjugated systems is 8 and 17 Å for Y₂-P₃₁ and Y₂-P₃₂, respectively (26)). Thus, the faster rate of Y₂ oxidation (30 ns to 50 μs) (70, 71) than the rate of Y₁ oxidation (~ms) (72, 73) by P680⁺ could be related to this asymmetric charge distribution, in addition to the difference in the proton transfer mechanism, i.e. Y₂ shifts a proton only to the neighboring D1-His-190 (57, 62–64), whereas a proton from Y₁ is transferred to the bulk through a long proton pathway (58, 65).

**Experimental procedures**

**Construction of a D1-V157H site-directed mutant**

Site-directed mutagenesis of the psbA2 gene encoding the D1 subunit in *Synechocystis* sp. PCC 6803 was performed following the method of Chu et al. (74) with some modifications. A psbA triple-deletion strain (ΔpsbA1/ΔpsbA2/ΔpsbA3) was generated from the *Synechocystis* sp. PCC 6803 47-H strain, which has a six-histidine tag at the C terminus of the CP47 subunit and was selected by co-expression of the kanamycin (Km) resistance gene (75). The entire coding regions of psbA1 (slr1181) and psbA3 (slr11867) were replaced with the chloramphenicol (Cm) and erythromycin (Em) resistance genes, respectively, and the coding region of psbA2 (slr1311), except for the first 2 bp, was replaced with the gentamicin (Gm) resistance gene (76). The psbA triple-deletion strain was isolated and maintained on BG-11 agar plates containing 5 μg/ml Km, 5 μg/ml Cm, 5 μg/ml Em, and 5 μg/ml Sm in the presence of 5 mM glucose and 10 μM DCMU under a continuous low-light condition. The genotype of the D1-V157H mutant was confirmed by PCR analysis and DNA sequencing. No trace of the wild-type psbA2 gene was detected in all cultures of the mutant strain.

**Construction of a D2-V156H site-directed mutant**

Site-directed mutagenesis of the psbD1 gene encoding the D2 subunit in *Synechocystis* sp. PCC 6803 was performed according to the method of Tang et al. (77) with some modifications. A psbD1/psbD2/psbC-deletion strain (ΔpsbD1/ΔpsbD2/ΔpsbC) was generated from the *Synechocystis* sp. PCC 6803 47-H strain (75). The entire coding regions of psbD1 (sll0849) and psbC (sll0851) were replaced with the Em resistance gene, although that of psbD2 (sll0927) was replaced with the Cm resistance gene. The psbD1/psbD2/psbC-deletion strain was isolated and maintained on BG-11 agar plates containing 5 μg/ml Km, 5 μg/ml Cm, 5 μg/ml Em, and 10 μM DCMU under a continuous low-light condition. The genotype of the strain was confirmed by PCR analysis.

The coding region of psbD1 and psbC together with the 585 bp upstream of the ATG start codon of psbD1 and the 589 bp downstream of the TAG stop codon of psbC in the genomic DNA was amplified by PCR using the following six primers, including five restriction enzyme sites: psbD1-1, 5'–GGA-TCTTCCAGAGATGATTTCCAACGCATCTCCTCAAAGCCG-3' (EcoRI site underlined); psbD1-2, 5′–CATATTGGAAGATGATTTCCAACGCATCTCCTCAAAGCCG-3’ (SalI site underlined); psbD1-3, 5′–GGGCTACTTTGCTGGACTGATCCTCCAATATGCTTTTCCAAC-3’ (SalI site underlined); psbD1-4, 5′–TATCCACTGGCATTGGATCATTGGCAGATCCTGCCATCATCAATTCC-3’ (SpeI site underlined); psbD1-5, 5′–CCTGACCTGGATCATTGGCAGATCCTGCCATCATCAATTCC-3’ (SpeI site underlined); psbD1-6, 5′–CTGCCCTGGCAGATCCTGCCATCATCAATTCC-3’ (SpeI site underlined); psbD1-7, 5′–GGA-TCTTCCAGAGATGATTTCCAACGCATCTCCTCAAAGCCG-3' (EcoRI site underlined); psbD1-8, 5′–GGGCTACTTTGCTGGACTGATCCTCCAATATGCTTTTCCAAC-3’ (SalI site underlined); psbD1-9, 5′–GGA-TCTTCCAGAGATGATTTCCAACGCATCTCCTCAAAGCCG-3’ (EcoRI site underlined); psbD1-10, 5′–GGGCTACTTTGCTGGACTGATCCTCCAATATGCTTTTCCAAC-3’ (SalI site underlined); psbD1-11, 5′–GGA-TCTTCCAGAGATGATTTCCAACGCATCTCCTCAAAGCCG-3’ (EcoRI site underlined); psbD1-12, 5′–GGA-TCTTCCAGAGATGATTTCCAACGCATCTCCTCAAAGCCG-3’ (EcoRI site underlined); psbD1-13, 5′–GGGCTACTTTGCTGGACTGATCCTCCAATATGCTTTTCCAAC-3’ (SalI site underlined); psbD1-14, 5′–GGGCTACTTTGCTGGACTGATCCTCCAATATGCTTTTCCAAC-3’ (SalI site underlined).
site underlined). The PCR fragments were cloned in the pMD19-T vector (TaKaRa) using the In-Fusion HD cloning kit (Clontech), and the resultant plasmid was designated as pRN105. The Sm resistance gene was inserted into the pRN105 at the BamHI site in the same direction as the pshbD1 and psbC genes. This plasmid, designated as pRN126, was used as a parental vector for site-directed mutagenesis. A wild-type control strain of the D2 subunit (D2-WT) was obtained by transforming the pshbD1/pshbD2/psbC-deletion strain with pRN126. D2-V156H mutation was introduced into pRN105 by replacing a GTC codon at the target site with a CAC codon using inverse PCR. The DNA fragment, which was obtained by digestion using Xbal and Sall, was inserted into pRN126 at the corresponding site. The resultant plasmid was introduced into the pshbD1/pshbD2/psbC-deletion strain. The D2-WT and D2-V156H strains were isolated and maintained on BG-11 agar plates containing 5 μg/ml Km, 5 μg/ml Cm, and 5 μg/ml Sm in the presence of 5 mM glucose and 10 μM DCMU under a continuous low-light condition. The genotype of the D2-V156H mutant was confirmed by PCR analysis and DNA sequencing. No trace of the wild-type pshbD1 gene was detected in all cultures of the mutant strain.

**Cell growth**

WT and mutant cells on the agar plate were inoculated into 40 ml of BG-11 medium (78) supplemented with 4 mM Hepes-NaOH (pH 7.5) and 5 μg/ml Km/Cm/Sm and Km/Cm/Sm for D1-WT/D1-V157H and D2-WT/D2-V156H, respectively, and were grown phototrophically by bubbling with air containing 1% (v/v) CO2 at 30 °C under continuous illumination (20 μmol photons m \(^{-2}\) s \(^{-1}\)) using white fluorescent lamps. Cells at this stage were used for the measurements of O2 evolution and TL glow curves. For PSII core preparation, cells were further grown in an 8-liter culture bottle without antibiotics under the phototrophic growth condition mentioned above. Cells cultured in six bottles (total volume of 48 liters) were used for preparation of PSII core complexes from each strain.

**Preparation of PSII core complexes**

PSII core complexes were purified according to the method by Sakurai et al. (79) with some modifications. Harvested cells were washed once with a buffer (pH 6.0) containing 50 mM Mes-NaOH, 5 mM CaCl\(_2\), 10 mM MgCl\(_2\), and 25% (w/v) glycerol (buffer A). The cells in buffer A were disrupted by glass beads as described previously (57), and then the lysate was diluted with an equal volume of a buffer (pH 6.0) containing 50 mM Mes-NaOH, 5 mM CaCl\(_2\), and 10 mM MgCl\(_2\). Unbroken cells were removed by centrifugation at 2000 × g for 5 min, and then the supernatant was centrifuged at 48,000 × g for 20 min, providing thylakoid membranes as resultant pellets. Thylakoids suspended in buffer A were solubilized with 1% (w/v) n-dodecyl β-D-maltoside (DM) at a Chl concentration of 1.0 mg/ml by stirring for 10 min on ice. After centrifugation at 27,000 × g for 15 min, the resultant supernatant was applied to a Ni\(^{2+}\) affinity column equilibrated with buffer A containing 0.04% DM (buffer B). The column was washed with 1 volume of buffer B containing 5 mM l-histidine and further washed with buffer B (pH 6.0) containing 100 mM NaCl and 100 mM imidazole-HCl until the eluate became colorless. PSII core complexes were eluted with buffer B containing 50 mM l-histidine and then concentrated by ultrafiltration (Vivaspin 20, Sartorius Stedim, 100-kDa molecular mass cutoff).

Manganese depletion from PSII was performed by treatment of PSII core complexes (0.35 mg Chl/ml) with 10 mM NH\(_4\)OH in a buffer (pH 6.5) containing 20 mM Mes-NaOH, 5 mM NaCl, and 0.03% DM (buffer C) for 1 h on ice in the dark. The sample was then washed with buffer C and concentrated to ~3.5 mg of Chl/ml using ultrafiltration (Vivaspin 500, Sartorius Stedim, 100-kDa molecular mass cutoff). For QA depletion, PSII core complexes in a buffer (pH 6.5) containing 200 mM Mes-NaOH, 5 mM NaCl, and 0.03% DM were treated with 100 mM sodium dithionite and 30 μM benzyl viologen (80), followed by dark incubation for 18 h at 4 °C. The manganese cluster was also removed during this treatment. The resultant QA-depleted PSII core complexes were washed with buffer C and concentrated to ~3.5 mg of Chl/ml using ultrafiltration.

**Measurement of O₂ evolution activity**

O₂ evolution activity was measured using a Clark-type oxygen electrode at 30 °C. Cells (10 μg of Chl) were suspended in BG11 medium containing 1 mM 2,6-dichloro-p-benzoquinone and 1 mM potassium ferricyanide as electron acceptors, and O₂ evolution was recorded upon illumination by saturating light.

**TL measurements**

WT and mutant cells were centrifuged at 1000 × g for 5 min at 25 °C and suspended in fresh BG-11 medium (0.25 mg of Chl/ml). The cells were then exposed to continuous light (200 μmol photons m \(^{-2}\) s \(^{-1}\); ~16 milliwatts cm \(^{-2}\) at the sample point) from a white fluorescent lamp for 30 s at 30 °C, followed by incubation at this temperature for 5 min in the dark. TL measurements were performed using a laboratory-built apparatus as described previously (81). A cell suspension (70 μl) in the presence of 50 μM DCMU was loaded onto a piece of filter paper and illuminated with continuous white light (~55 milliwatts cm \(^{-2}\) at the sample point) from a halogen lamp (MEJIRO PRECISION PHL-150) for 10 s at ~20 °C. The sample was quickly cooled down and then warmed at a rate of 40 °C/min to record a TL glow curve.

**FTIR measurements**

Light-induced FTIR difference spectra were recorded using a Bruker VERTEX 80 spectrophotometer equipped with an MCT detector (InfraRed D313-L) at 4 cm \(^{-1}\) resolution. A Ge filter to cut IR light at >2200 cm \(^{-1}\) (Andover, 4.50ILP-25) was placed in the IR path in front of the sample to improve the signal-to-noise ratios of spectra as well as to block a He-Ne laser beam from the interferometer. P680+/P680 FTIR spectra were measured following the method described previously (22). An aliquot (3.5–5 μl) of the QA-depleted PSII suspension was mixed with 1 μl of 500 mM potassium ferricyanide and 1 μl of 10 mM SiMo on a CaF\(_2\) plate (13 mm in diameter). The sample was lightly dried under N\(_2\) gas flow and covered with another CaF\(_2\) plate with 0.7 μl of water. The sample temperature was adjusted to 250 K in a liquid-N\(_2\) cryostat (Oxford, model DN1704) using a tempera-
Asymmetric charge distribution on P680$^+$ in PSII

ture controller (Oxford, model ITC-5). Single-beam spectra with two scans (1-s accumulation) were recorded under dark and during illumination. This measurement was repeated 5000 times, and average single-beam spectra were used to calculate a P680$^+$/P680 difference spectrum as a light-minus-dark difference. Light illumination was performed with a continuous-wave beam at 661 nm (~38 milliwatts cm$^{-2}$ at the sample point) from a diode laser (L4660S-90-TE, Micro Laser Systems).

$Y_Z/Y_Z$ FTIR spectra were measured as described previously (57). An aliquot (4–5 μl) of the manganese-depleted PSII sample was mixed with 1 μl of 100 mM potassium ferricyanide on a BaF$_2$ plate (13 mm in diameter). The sample was lightly dried under Nz gas flow and covered with another BaF$_2$ plate with 0.7 μl of water. The sample temperature was adjusted to 250 K in the cryostat. Single-beam spectra with 50 scans (25-s accumulation) were recorded twice before and once after single-flash illumination. The sample was then dark-adapted for 225 s. This measurement scheme was repeated 80 times, and average spectra were used to calculate a $Y_Z/Y_Z$ difference spectrum as light-minus-dark difference and a base line as a light-minus-dark difference representing a noise level. Flash illumination was performed with a Q-switched Nd:YAG laser (Quanta-Ray INDI-40-10; 532 nm, ~7 ns full width at half-maximum) with a power of ~7 mJ pulse$^{-1}$ cm$^{-2}$ at the sample point.

$Y_O/Y_O$ FTIR spectra were measured with a method described previously (58). An aliquot (3 μl) of the manganese-depleted PSII was mixed with 1 μl of 20 mM potassium ferricyanide and 1 μl of 20 mM potassium ferrocyanide on a BaF$_2$ plate (25 × 25 mm). The sample was lightly dried under Nz gas flow. The resultant sample film in an oval shape ($6 \times 9$ mm) was moderately hydrated by sealing the sample under BaF$_2$ plate and a silicone spacer (0.5 mm in thickness) enclosing 2 μl of 40% (v/v) glycerol solution without touching the sample (82). The sample temperature was adjusted to 10°C by circulating cold water in a copper holder. Single-beam spectra with 100 scans (50-s accumulation) were recorded twice before and once after five flashes (1 Hz) from the Nd:YAG laser. The sample was then dark-adapted for 750 s. This measurement scheme was repeated 50 times, and average single-beam spectra were used to calculate a $Y_O/Y_O$ difference spectrum as a light-minus-dark difference and a base line as a dark-minus-dark difference representing a noise level.

**QM/MM calculation of P680 models**

QM/MM calculations were performed following the method described previously (83, 84). The initial coordinates of PSII models were obtained from the X-ray structure at a 1.9 Å resolution (Protein Data Bank code 3ARC) (26). In addition to P$_{D1}$ and P$_{D2}$ cofactors (Chl$_{D1}$, Chl$_{D2}$, Pheo$_{D1}$, and Pheo$_{D2}$), amino acid residues, and water molecules located within 10 Å from the heavy atoms of P$_{D1}$ and P$_{D2}$ (including the phytol chains) were extracted from the X-ray structure (the whole QM/MM region is shown in Fig. 6). Hydrogen atoms of amino acid residues were generated and optimized using the AMBER force field (85), whereas those of Chl and Pheo molecules were originally produced and optimized by the DFT method with the B3LYP functional using 6–31G(d) as a basis set. Atomic charges of these molecules were also calculated as electrostatic potentials. QM/MM calculations were performed using the two layer ONIOM method (86) with the electronic embedding scheme in the Gaussian 09 program package (87). The QM region consists of P$_{D1}$ and P$_{D2}$ (without the phytol chains), and the side chains of D1-His-198, D2-His-197, D1-Val-157, and D2-Val-156 (Fig. 7A). For the models of D1-V157H and D2-V156H mutants, D1-Val-157 and D2-Val-156, respectively, were replaced with a His side chain (Fig. 7, B and C). As an initial structure, a His side chain (neutral N$_x$H or N$_x$H form) was positioned so that the NH group interacts with the 13$^v$-keto C=O of P$_{D1}$ (29). In the QM/MM geometry optimization, the coordinates of the QM region were fully relaxed, whereas those of the MM region were fixed. Geometry optimization and normal mode calculations of the QM region were performed using the DFT method at the B3LYP/6–31G(d) level. Calculated 13$^v$-keto C=O frequencies were scaled using a scaling factor of 0.9416 to adjust the calculated value of the P$_{D1}$ C=O (1808.6 cm$^{-1}$) to the experimental one of 1703 cm$^{-1}$ obtained from the $Y_Z/Y_Z$ difference spectrum (Table 2).

Author contributions—R. N. and T. N. designed the study and wrote the manuscript. R. N., M. Y., and H. U.-N. performed the experiments, and S. N. performed QM/MM calculations. All authors reviewed the results and approved the final version of the manuscript.

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