Environment of Tyr$_Z$ in Photosystem II from *Thermosynechococcus elongatus* in which PsbA2 Is the D1 Protein

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The main cofactors that determine the photosystem II (PSII) oxygen evolution activity are borne by the D1 and D2 subunits. In the cyanobacterium *Thermosynechococcus elongatus*, there are three *psbA* genes coding for D1. Among the 344 residues constituting D1, there are 21 substitutions between PsbA1 and PsbA3, 31 between PsbA1 and PsbA2, and 27 between PsbA2 and PsbA3. Here, we present the first study of PsbA2-PSII. Using EPR and UV-visible time-resolved absorption spectroscopy, we show that: (i) the time-resolved EPR spectrum of Tyr$_Z$ phenol and its environment, likely the Tyr-O$^\cdot$ bond, is slightly modified; (ii) the split EPR signal arising from Tyr$_Z$ in the (S$_3$Tyr$_Z$)$'$ state induced by near-infrared illumination at 4.2 K of the S$_3$Tyr$_Z$ state is significantly modified; and (iii) the slow phases of P$_{680}^+$ reduction by Tyr$_Z$ are slowed down from the hundreds of ms time range to the ms time range, whereas both the S$_1$Tyr$_Z$ $\rightarrow$ S$_2$Tyr$_Z$ and the S$_2$Tyr$_Z$ $\rightarrow$ S$_3$Tyr$_Z$ + O$_2$ transition kinetics remained similar to those in PsbA(1/3)-PSII. These results show that the geometry of the Tyr$_Z$ phenol and its environment, likely the Tyr-O$^\cdot$-$\cdot$-His-190 bonding is an important parameter for PSII activity.

**Background:** Photosystem II, the water-splitting enzyme, includes a protein, D1, which can be coded by three different *psbA* genes in *Thermosynechococcus elongatus*.

**Results:** In PsbA2-PSII, the environment of Tyr$_Z$ is different from that in PsbA1-PSII and PsbA3-PSII.

**Conclusion:** The geometry of the Tyr$_Z$-O$^\cdot$-$\cdot$-His-190 bonding is an important parameter for PSII activity.

**Significance:** The environment of the cofactors is involved in the tuning of the electron transfer efficiency.
The Mn₄CaO₅ cluster acts both as a device accumulating oxidizing equivalents and as the catalytic site for water oxidation. The enzyme cycles sequentially through five redox states, denoted Sₙ, where n stands for the number of oxidizing equivalents stored. Upon formation of the Sₐ state, two molecules of water are rapidly oxidized, the Sₐ state is regenerated, and O₂ is released (10, 11).

Cyanobacterial species have multiple psbA variants coding for the D1 protein (e.g. Refs. 12–20). These different genes are known to be differentially expressed depending on the environmental conditions (e.g. Refs. 12–18). In particular, specific up/down-regulations of one of these genes under high light conditions is indicative of a photo-protection mechanism. For example, the mesophilic cyanobacterium, Synechocystis PCC 6803, has three psbA genes. Two of these (psbAII and psbAIII) produce an identical D1. Nevertheless, although psbAII is expressed under the “normal” cultivation conditions, transcription of psbAIII is induced by high light or UV light (15), and that of psbAII seems triggered by microaerobic conditions (18). T. elongatus also has three different psbA genes in its genome (20). The comparison of the mature D1 amino acid sequence deduced from the psbA₃ gene with those of the psbA₁ and psbA₂ genes points to a difference of 21 and 31 residues, respectively (see supplemental Figs. S1 and S2). It has been reported that in T. elongatus, psbA₁ is constitutively expressed under normal laboratory conditions, whereas the transcription of psbA₃ occurred under high light or UV light conditions (16, 21, 22).

In T. elongatus, the change of Q130 in PsbA1-PSII to E130 in PsbA3-PSII has been shown to increase the midpoint potential of PheoD1/PheoD1 by 17 mV from −522 mV in PsbA1-PSII (23) to −505 mV in PsbA3-PSII (19). Because this increase was half the one observed upon single site-directed mutagenesis in Synechocystis PCC 6803 (24, 25), this led us to propose that the effects of the D1-Q130E substitution could be, at least partly, compensated for by some of the additional amino acid changes associated with the PsbA3 for PsbA1 substitution (19). For manganese-depleted PSII and above pH 7.5, where the phenolic groups of PheoD1/PheoD1, TyrZ, and TyrF are deprotonated (26), and the electron transfer rate between P₆₈₀° and TyrZ has been found to be slightly faster in PsbA3-PSII (global t₁/₂ ~100 μs) than in PsbA1-PSII (global t₁/₂ ~200 μs) (19). The temperature dependences of the S₂Qₐ⁻ charge recombination in PsbA1 and PsbA3 have shown that the environments of Qₐ and, as a consequence, its redox potential, are likely to be different (19). The exchange of S270 in PsbA1 for A270 in PsbA3 has been suggested to influence the stabilization of the sulfoquinovosyl-diacetylglycerol molecule that lies between Qₐ and nonheme iron (27). Maybe as a consequence, the binding of bromoxynil in PsbA3-PSII and in PsbA1-PSII has been found to differ, suggesting that the Qₐ pocket had different properties. It has been also found that the midpoint potential of the Fe³⁺/Fe⁴⁺ couple was likely higher in PsbA1-PSII than in PsbA3-PSII (28). In addition, under photo-inhibitory conditions, the accelerated decrease in O₂ evolution in WT*1 ⁴ producing PsbA1-PSII) cells was found to correlate with a much faster inhibition of the Sₐ state formation than in WT*3 (producing PsbA3-PSII) cells (29).

Although there have been an increasing number of studies aimed at characterizing the properties of PsbA1-PSII when compared with those of PsbA3-PSII, those of PsbA2-PSII have not yet been reported. In the present work, we describe the first construction of a T. elongatus deletion mutant lacking both the psbA₁ and the psbA₃ genes and expressing only psbA₂. We focused our first characterization of PsbA2-PSII on the electron transfer reactions involving TyrZ. Using continuous wave EPR at helium temperature, time-resolved EPR at room temperature, and time-resolved UV-visible absorption spectroscopy, it is shown that the properties of TyrZ are modified in PsbA2-PSII when compared with those in Psb(A1/3)-PSII.

**EXPERIMENTAL PROCEDURES**

**Construction of T. elongatus Mutants—**The construction of the ΔpsbA₁ΔpsbA₂ T. elongatus deletion mutant (WT*3) from a T. elongatus 43-H strain that had a His₅ tag on the C terminus of CP43 (32) has been previously described in Ref. 33.

For making the ΔpsbA₁ΔpsbA₂ T. elongatus deletion mutant (WT*2) (Fig. 1), first, the psbA₁ gene and its promoter region (~180 bp) were substituted together from the 43-H strain with a chloramphenicol-resistant cassette (~1300 bp) by using the plasmid vector pΔpsbA₁. Then, the psbA₃ gene was substituted with a spectinomycin/streptomycin resistance gene cassette (~2100 bp) by using the plasmid pΔpsbA₃. For construction of pΔpsbA₂, a DNA fragment of ~2300 bp of the psbA₂ gene (tlr1844) including its promoter region (~180 bp) and the 3’-flanking region of psbA₂ (~1000 bp) was cloned from T. elongatus wild-type genomic DNA by PCR amplification and subcloned into a plasmid vector pBluescript II SK+ at EcoRV and Xhol sites. Next, a chloramphenicol resistance gene cassette (~1300 bp) was ligated to the upstream of the psbA₂ gene at BamHI and EcoRV of the plasmid DNA. Then, a separately amplified ~900-bp DNA fragment of the 3’-flanking region of psbA₂ (but without the psbA₂ promoter region) was ligated to the subcloned plasmid vector at SacI and BamHI. For the construction of pΔpsbA₂, a DNA fragment of ~900 bp of the 3’-flanking region of the psbA₂ (tlr1477) was cloned from T. elongatus wild-type genomic DNA by PCR amplification and subcloned into a plasmid vector pBluescript II SK+ between SacI and EcoRI sites. Then, a spectinomycin/streptomycin resistance gene cassette (~2100 bp) was inserted at PstI and SacI. Then, a separately amplified ~1100-bp DNA fragment of the 5’-flanking region of psbA₂ was ligated to the subcloned plasmid vector at PstI and PstI.

The T. elongatus transformants were selected as single colonies on DTN agar plate containing appropriate antibiotics (25 μg ml⁻¹ spectinomycin, 10 μg ml⁻¹ streptomycin, 40 μg ml⁻¹ kanamycin, and 5 μg ml⁻¹ chloramphenicol). Segregation of all genome copies was confirmed by difference in length of amplified DNA by PCR using the P1 primer (5’-GCTGTACTGCCATCGCTGGGCCACCACCTG-3’) and P2 primer (5’-GGAC-TTATCACTTATACTAGAGGGTTG-3’) for psbA₁–psbA₂ region, and using the P3 primer (5’-GGTTGGATCCACGCCCAGCGATCGCGGAG-3’) and P4 primer (5’-CCA-
TGCCC CGAAACAGC-3') for psbA3 region as shown in Fig. 1. Complete segregation of the deletion mutants was confirmed by PCR amplification as shown in Fig. 1D. In the wild type, a 3900-bp DNA fragment including both psbA1 and psbA2 was amplified by P1 and P2 primers (lane 1). In contrast, a 4000-bp fragment (lane 2) and a 2850-bp fragment (lane 3) were amplified with using the same combination of the primers in WT*1 and WT*3, respectively. In the region of psbA3, a 2200-bp fragment was amplified with P3 and P4 primers in both wild-type and WT*3 genomes (lanes 5 and 7), whereas a 3100-bp fragment was amplified in WT*2 genome (lane 6).

Purification of PSII—PSII were purified with the protocol already described (28). PSII samples were suspended in 1 M betaine, 10% glycerol, 15 mM CaCl2, 15 mM MgCl2, 40 mM MES, pH 6.5 adjusted with NaOH. For the low temperature X-band EPR experiments, glycerol was omitted because its presence decreases the yield of the near-infrared induced split EPR signal in the S3 state.

For manganese depletion, PSII samples were diluted ~10-fold in a medium containing 1.2 M Tris-HCl (pH 9.2) and were incubated under room light at 4 °C for 1 h. The samples were collected by centrifugation (15 min at 170,000 × g) after the addition of 1.2 M Tris (pH 9.2) containing 50% (w/v) polyethylene glycol 8000 so that the final PEG concentration was 12%. The pellet was resuspended in a medium containing 1 M betaine, 10% glycerol, 15 mM CaCl2, 15 mM MgCl2, 40 mM MES, pH 6.5 adjusted with NaOH, and 12% PEG. After a new centrifugation, the pellet was resuspended in 1 M betaine, 10% glycerol, 15 mM CaCl2, 15 mM MgCl2, 40 mM MES, pH 6.5 adjusted with NaOH.

Oxygen Evolution Measurements—Oxygen-evolving activity of purified PSII (5 μg of Chl ml⁻¹) was measured under continuous saturating white light at 25 °C by polarography using a Clark type oxygen electrode (Hansatech). A total of 0.5 mM 2,6-dichloro-p-benzoquinone (dissolved in dimethyl sulfoxide) was added as an electron acceptor.

EPR Spectroscopy—For helium temperature measurements, continuous wave EPR spectra were recorded with a Bruker Elexys 500 X-band spectrometer equipped with a standard ER 4102 (Bruker) X-band resonator, a Bruker teslameter, an Oxford Instruments cryostat (ESR 900), and an Oxford ITC504 temperature controller. Flash illumination at room tempera-
ture was provided by a neodymium:yttrium-aluminum garnet laser (532 nm, 550 mW, 8-ns Spectra Physics GCR–230–10). PSII samples at 1.1 mg of Chl ml$^{-1}$ were loaded in the dark into quartz EPR tubes and dark-adapted for 1 h at room temperature. Then, the samples were synchronized in the $S_1$ state with one pre-flash (34). After a further 1-h dark adaptation at room temperature and the addition of 0.5 mM PPBQ dissolved in dimethylsulfoxide, the samples were either frozen immediately to 198 K in a solid CO$_2$/ethanol bath or illuminated by one or two additional flashes to generate the $S_2$ and $S_3$ states before being frozen in the dark to 198 K and then transferred to 77 K. In both cases, the samples were degassed at 198 K prior to the recording of the spectra.

For time-resolved measurements at room temperature, the spectrometer was equipped with a Super High Quality Bruker cavity. Saturating laser flash illumination at room temperature was provided by the laser described above. PSII at 1.1 mg of Chl ml$^{-1}$ was loaded into a small volume flat cell (100 μl) in the presence of 0.5 mM phenyl-$p$-benzoquinone (PPBQ) and 1 mM potassium ferricyanide. Ferricyanide was added to avoid any contamination from the PPBQ$^-$ signal, which is detectable in the hundred μs time range after the flash illumination in the absence of ferricyanide. Formation and decay of the signal following laser flash illumination were measured at 32 magnetic field positions spread over 50 G and centered on the Tyr$Z^-$ EPR signal. For each of the 32 magnetic field values, 16 scans were averaged. The two-dimensional spectra (time versus field) of ~12–16 samples were averaged. Half of the two-dimensional spectra were obtained by increasing the magnetic field, and the other half were obtained by decreasing the magnetic field. When indicated, near-IR illumination of the samples was done directly in the EPR cavity and was provided by a laser diode emitting at 820 nm (Coherent, diode S-81–1000C) with a power of 600–700 milliwatts at the level of the sample.

The High Field-EPR measurements were taken on a locally built spectrometer described previously (35). Using a manganese-doped magnesium oxide sample, we verified that the relative accuracy of the magnetic field was better than 1 millitesla in the field ranges used in this study. The microwave frequency was accurate to better than 1 MHz. Hence, the measurement accuracy in $g$ was expected to be $1 \times 10^{-4}$.

UV-visible Absorption Change Spectroscopy—Absorption changes were measured with a lab-built spectrophotometer (36) where the absorption changes are sampled at discrete times by short flashes. These flashes were provided by a neodymium:yttrium-aluminum garnet (355 nm) pumped optical parametric oscillator, which produces monochromatic flashes (1 nm full-width at half-maximum) with a duration of 5 ns. Excitation was provided by a second neodymium:yttrium-aluminum garnet (532 nm) pumped optical parametric oscillator, which produces monochromatic saturating flashes at 700 nm (1 nm full-width at half-maximum) with a duration of 5 ns. The path length of the cuvette was 2.5 mm. PSII was used at 25 μg of Chl ml$^{-1}$ in 10% glycerol, 1 mM betaine, 15 mM CaCl$_2$, 15 mM MgCl$_2$, and 40 mM MES (pH 6.5). PSII was dark-adapted for ~1 h at room temperature (20–22 °C) before the additions of 0.1 mM PPBQ dissolved in dimethyl sulfoxide. For kinetic measurements, the time delay between the ac tinic flash and the detector flash was first increased from the smaller value to the larger value and then varied in the opposite direction. For each time delay, the measurements were repeated four times so that each data point is the average of eight measurements. The traces shown are typical of those obtained with at least three different PSI preparations.

**RESULTS**

The oxygen evolution activity of purified PsbA2-PSII was 3000–3500 μmol of O$_2$ (mg of Chl)$^{-1}$ ms$^{-1}$. This activity is close to that found for PsbA1-PSII and about half of that commonly found for PsbA3-PSII (33).

Fig. 2 shows the amplitude of the absorption changes associated with each flash in a series with PsbA3-PSII (circles) and with PsbA2-PSII (squares). Measurements were done at 292 nm (37–39) and at 200 ms after the flashes, i.e. after completion of the reduction of Tyr$Z^-$ by the water-oxidizing complex. At this wavelength, the reduction of PPBQ does not lead to any absorption changes, and the successive oxidation steps of the water-oxidizing complex have significant extinction coefficients (38). The pattern, oscillating with a period of four, is clearly observed for both types of PSII preparations with very similar amplitude on the first flash. However, the damping is larger in PsbA2-PSII than in PsbA3-PSII. Indeed, the maxima are clearly shifted from the 5th, 9th, 13th, etc., flashes in PsbA3-PSII to the 6th, 10th, 14th, etc., flashes in PsbA2-PSII. This is at variance with the PsbA(1/3) cases, which displayed similar period four oscillation characteristics like the miss parameter and $S_1/S_0$ ratio in dark-adapted material (33, 39, 40). This suggests that functional differences exist between PsbA2-PSII and PsbA(1/3)-PSII.

To determine which step(s) is(are) kinetically affected and therefore responsible for the larger miss parameter in PsbA2-PSII, we first measured the absorption changes at 292 nm in the 10 μs to ms time ranges after the first three flashes in a series to assess the kinetics of electron transfer associated with the $S_1$$\rightarrow S_1$,$S_1$$\rightarrow S_2$$\rightarrow S_2$,$S_2$$\rightarrow S_3$ transitions in both the PsbA2-PSII and the PsbA3-PSII. At 292 nm, the absorption changes associated with the $S_2$$\rightarrow S_3$ transition are small and preclude a reliable kinetic analysis. As shown in Fig. 3, we did not observe any significant differences for the kinetics of the absorption changes associated
with the $S_1$, $TyrZ$ → $S_2$, $TyrZ$ and $S_3$, $TyrZ$ → $S_3$, $TyrZ$ transitions in PsbA2-PSII (squares) when compared with PsbA3-PSII (circles). Thus, the larger miss parameter in PsbA2-PSII does not originate from a longer lifetime of the $S_i$, $TyrZ$ states. This period four oscillation pattern in the P680+ excited state, like uncoupled Chl*, for example. In this spectral region, the redox changes of several species, such as the Chls, cytochromes, $TyrZ$, and QA, could potentially contribute to the absorption changes. The most prominent ones, however, are those associated with the formation of P680+. The $P_{680}$+$P_{680}$ difference spectrum is characterized by a strong Soret band bleaching. After one flash, i.e., in the $S_1$ $P_{680}$+ state, the maximum of the bleaching was observed at 433 nm. After the second flash and third flash, i.e., in the $S_2$ $P_{680}$+, $S_3$ $P_{680}$+ state, the width of the bleaching increased, and the red-most parts of the spectra were slightly red-shifted when compared with the spectrum of the $S_1$ $P_{680}$+ state. The red-shift was reversed after the fourth and fifth flashes, i.e., in the $S_3$ $P_{680}$+ and $P_{680}$+ states. This period four oscillation pattern in the $P_{680}$+$P_{680}$ spectrum likely originates from an electrostatic effect on the $P_{D1}$+$P_{D2}$↔$P_{D1}$+$P_{D2}$ equilibrium due to the charge(s) stored on/around the Mn$_4$CaO$_5$ cluster (e.g., Ref. 4). Irrespective of the flash number, the difference spectra were similar to those in PsbA1-PSII (40) and PsbA3-PSII (33), thus showing that the distribution of the cation over the $P_{D1}$ and $P_{D2}$ chlorophylls is similar in both cases.

We conclude from the above data that the larger miss parameter is not due to a longer lifetime of any of the $S_i$, $TyrZ$ states that would result in a larger charge recombination probability. This makes the electron transfer step between $P_{680}$+ and $TyrZ$ the next candidate. Fig. 5A shows the time-resolved flash-induced absorption changes around 433 nm after each of the first five flashes applied to dark-adapted PsbA2-PSII (first flash, black; second flash, blue; third flash, red; fourth flash, green; fifth flash, orange). The spectra were recorded 20 ns after the flashes to avoid any spectral distortions due to the short lived excited state, like uncoupled Chl*, for example. In this spectral region, the redox changes of several species, such as the Chls, cytochromes, $TyrZ$, and QA, could potentially contribute to the absorption changes. The most prominent ones, however, are those associated with the formation of P680+. The $P_{680}$+$P_{680}$ difference spectrum is characterized by a strong Soret band bleaching. After one flash, i.e., in the $S_1$ $P_{680}$+ state, the maximum of the bleaching was observed at 433 nm. After the second flash and third flash, i.e., in the $S_2$ $P_{680}$+, $S_3$ $P_{680}$+ state, the width of the bleaching increased, and the red-most parts of the spectra were slightly red-shifted when compared with the spectrum of the $S_1$ $P_{680}$+ state. The red-shift was reversed after the fourth and fifth flashes, i.e., in the $S_3$ $P_{680}$+ and $P_{680}$+ states. This period four oscillation pattern in the $P_{680}$+$P_{680}$ spectrum likely originates from an electrostatic effect on the $P_{D1}$+$P_{D2}$↔$P_{D1}$+$P_{D2}$ equilibrium due to the charge(s) stored on/around the Mn$_4$CaO$_5$ cluster (e.g., Ref. 4). Irrespective of the flash number, the difference spectra were similar to those in PsbA1-PSII (40) and PsbA3-PSII (33), thus showing that the distribution of the cation over the $P_{D1}$ and $P_{D2}$ chlorophylls is similar in both cases.

FIGURE 3. Kinetics of absorption changes at 292 nm after first flash (red), second flash (blue), and third flash (black) given to dark-adapted PsbA3-PSII (circles and continuous lines) or PsbA2-PSII (squares and dashed lines). Other experimental conditions were similar to those in Fig. 2.

FIGURE 4. Light-minus-dark EPR spectra induced by either one flash (A) or two flashes (B) at room temperature in presence of 0.5 mM PPBQ and recorded on PsbA3-PSII (spectrum a, black) or PsbA2-PSII (spectrum b, red). Sample concentration was 1.1 mg of Chl ml$^{-1}$. Instrument settings were: modulation amplitude, 25 G; microwave power, 20 milliwatt; microwave frequency, 9.5 GHz; modulation frequency, 100 kHz; and temperature, 8.5 K. The central part of the spectra corresponding to the $TyrZ$ region was deleted.
Fig. 5B shows the decay of $P_{680}^+$ measured at 433 nm after each of the first three flashes in PsbA3-PSII (filled circles) and PsbA2-PSII (open circles). After the first flash (black symbols), both the tens of ns and the tens of $\mu$s phases were found comparable in PsbA3-PSII and PsbA2-PSII in terms of amplitude and $\tau_{\text{ns}}$. After the second flash (blue symbols) and the third flash (red symbols), the $P_{680}^+$ decay was much slower in PsbA2-PSII, particularly in the hundreds of $\mu$s time domain. This shows up even more clearly after averaging the decay traces from the 1st flash to the 20th flash (supplemental Fig. S6).

The finding that the reduction kinetics of $P_{680}^+$ is hardly affected on the first flash shows that possible changes of the properties of the electron acceptor side originating from the PsbA(1/3) to PsbA2 substitution did not significantly increase the percentage of centers in which the $P_{680}^+$ reduction measured at 433 nm after the first three flashes in PsbA3-PSII (filled circles) and PsbA2-PSII (open circles). Black circles, first flash; blue circles, second flash; red circles, third flash.

According to the current understanding of the multiphasicity of the reduction of $P_{680}^+$, the ns components are kinetically limited by the electron transfer process, whereas the $\mu$s phases involve proton-coupled transfer reactions (e.g. Refs. 43–45). In this framework, the present data would thus point to a slower proton transfer process in PsbA2-PSII. Two amino acid substitutions on the electron donor side of PsbA2-PSII may affect the orientation of the helices, which respectively bear His-190 and TyrZ; the C144P and P173M exchanges. These two substitutions may impact the hydrogen bond between TyrZ and His-190 and/or the H-bond network in which these two residues are involved. If such is indeed the case, this would be expected to affect the rates of the proton transfer steps associated with the oxidation of TyrZ. This was assessed using EPR spectroscopy, which has been shown to probe the geometry and the environment of the TyrZ phenol ring (e.g. Refs. 46 and 47).

In the $S_3$ state, NIR illumination at $\sim$4 K results in the formation of a split EPR signal (48, 49), attributed to a $(S_2TyrZ^{'})$ state formed by NIR-induced conversion of the manganese cluster into an “activated” state able to oxidize TyrZ and thus leading to the formation of $(S_1TyrZ^{'})$ at the expense of the $S_2TyrZ$ state (50). This split signal is attributed to the magnetic interaction between TyrZ, with a spin state $S = 1/2$ and the Mn$_4$CaO$_5$ cluster possibly in a $S = 7/2$ spin state (51), and as such is very sensitive to the geometry of the TyrZ/Mn$_4$CaO$_5$ ensemble. As an example of this sensitivity, the split EPR spectrum is significantly modified upon the Ca$^{2+}$/Sr$^{2+}$ exchange (52). Importantly, these modifications can be reliably ascribed to changes in the geometry of the bridge between TyrZ and Ca$^{2+}$/Sr$^{2+}$ via a water molecule (3) rather than to the alteration of the Mn$_4$CaO$_5$ magnetic structure, which has been independently shown to be only slightly affected by the Ca$^{2+}$/Sr$^{2+}$ exchange (41).

Fig. 6 shows the EPR difference spectra after-minus-before near-infrared illumination in PsbA2-PSII (red spectrum), which are compared with that recorded in PsbA3-PSII (black spectrum). Notably, either PsbA1-PSI or PsbA3-PSII can be used as control samples because their split signals are identical (40, 49, 52). Fig. 6 evidences manifest differences between the two samples. Because the PsbA exchange does not modify the EPR properties of the Mn$_4$CaO$_5$ cluster, at least in the $S_2$ and $S_3$ states, the changes in the split signal likely arise from a change in the EPR properties of TyrZ or in the magnetic interaction between TyrZ and the Mn$_4$CaO$_5$ cluster. The EPR properties of TyrZ that may be modified are: (i) the values and localization of the Mn$_4$CaO$_5$ cluster possibly in a $S = 1/2$ and the $S_2TyrZ$ state (50).
the spin densities on the carbons and oxygen bearing this spin density; (ii) the orientation of the \( \beta \)-methylene group versus the plan of the phenol ring; (iii) the \( g_x \), \( g_y \), and \( g_z \) values; (iv) the relative orientation of Tyr\( Z \) versus the Mn\(_4\)CaO\(_5\) cluster; and (v) the distance between Tyr\( Z \) and the Mn\(_4\)CaO\(_5\) cluster. The changes in the magnetic interaction between Tyr\( Z \) and the Mn\(_4\)CaO\(_5\) cluster could be assessed by a theoretical approach. Nevertheless, although some of the split signals, in acetate-treated PSII or in the \( S_0 \)Tyr\( Z \) generated at 4 K, have been successfully simulated (53, 54), the magnetic properties of the Mn\(_4\) moiety are poorly understood in the \( S_3 \) state, which precludes here a reliable simulation. Thus, to gain further insights into the structural reasons underlying these spectroscopic changes, we attempted to measure directly the EPR spectrum or Tyr\( Z \).

Figs. 7-9 report the results of time-resolved EPR experiments performed at room temperature. The time resolution of our EPR spectrometer is in the same time range as the lifetime of \( S_3 \)Tyr\( Z \) in WT \( T.\ elongatus \) PSII (\( t_{1/2} \sim 1 \text{ ms} \)). To circumvent this limitation, Ca\(^{2+}\) and Cl\(^-\) were substituted by Sr\(^{2+}\) and Br\(^-\) in PsbA3-PSII because it has been shown that this markedly increases the lifetime of \( S_3 \)Tyr\( Z \) (39). In such conditions, the \( S_3 \)Tyr\( Z \) to \( S_0 \)Tyr\( Z \) transition occurs with a \( t_{1/2} \) close to 7 ms (39), i.e. in a time domain compatible with the reliable detection of Tyr\( Z \)'s decay with our EPR set-up. Because in separate experiments (not shown) we checked that the Cl\(^-\)/Br\(^-\) exchange had no effect on the Tyr\( Z \)' spectrum and because the Ca\(^{2+}\)/Sr\(^{2+}\) exchange alone proved sufficient to allow us the full detection of the Tyr\( Z \) spectrum, the formation and decay of the Tyr\( Z \) signals following laser flash illumination were done in Sr\(^2+\)-containing PsbA2-PSII. These measurements were done at 32 magnetic field positions spread over 50 G and centered on the Tyr\( Z \)' EPR spectrum.

To validate the approach, we first applied the method to manganese-depleted PSII in which the lifetime of Tyr\( Z \) is much longer. Fig. 7A shows the results of such experiments, i.e. a two-dimensional spectrum (time versus field). Fig. 7B shows two slices extracted from the two-dimensional spectrum. The first one (black spectrum), before the flash, corresponds to the Tyr\( D \) spectrum, and the second one (red spectrum), immediately after the flash and after subtraction of the baseline for each magnetic field value, corresponds to the Tyr\( Z \) spectrum. Although the magnetic field resolution is here limited to 50/31 \( \sim 1.6 \) G, the Tyr\( D \) and Tyr\( Z \) spectra thus obtained are similar to those reported in the literature for manganese-depleted PSII (e.g. Refs. 46 and 55).

Fig. 8A shows the results of a similar two-dimensional experiment performed in Sr/Br-containing PsbA3-PSII. Here, in contrast to the situation prevailing in manganese-depleted PSII, the Tyr\( Z \) signal was kinetically detectable only in the
 PsiI. In addition, we did not find any significant changes in the differences between the PsbA2-PSII and PsbA3-PSII TyrZ detected in PsbA3-PSII (in vitro) for PsbA3-PSII (in Sr-containing PsbA2-PSII. Fig. 9 compares the TyrZ spectrum occurring while it decays (see supplemental Figs. S3 and S4).

The C144P and P173M exchanges are two of the less conservative differences between PsbA2 and PsbA1/3. In PsbA1 and PsbA3, the C144 is located in helix C on the periplasmic side just before the α-helix bearing TyrZ (the Y161), and the P173 is located in the loop between the C and D helices bearing TyrZ and the H190 (3) (see supplemental data). Many studies have documented the role and importance of this H-bond in determining the reaction pathway as well as the rate of the proton-coupled electron transfer process involved associated with the oxidation of TyrZ (e.g. Refs. 26, 30, and 31). Because its backbone dihedral angle is locked, proline is a singularly rigid amino acid. Its substitution for and by other residues in the vicinities of TyrZ and H190 is expected to have significant structural consequences on the local environment of TyrZ and therefore on this proton-coupled electron transfer. Therefore, it seems very likely that these substitutions have some consequences on the relative orientation of the TyrZ versus H190 and are therefore responsible for the changes reported in this work.

The period four oscillating pattern in PsbA2-PSII indicates that the miss parameter is larger than in either PsbA3-PSII (33, 39) or PsbA1-PSII (40) (Fig. 2). However, the yield of S2 formation estimated from the amplitude of the S2 EPR multiline signal was identical in PsbA2-PSII and PsbA3-PSII, thus showing that the yield of the S1 to S2 transition is not significantly affected (Fig. 4). In contrast, the smaller amplitude of the absorption change detected upon the second flash and the smaller S3 EPR signal and the larger fraction of remaining S2 multiline signal detected after two flashes applied to PSII centers synchronized in the S1 state prior to the flash illumination suggest that in PsbA2-PSII, the miss parameter strongly increases in the S2 to S3 transition. The fact that the miss parameter depends on the S state transitions decreases the accuracy of a fitting of the period four oscillations in Fig. 2 (nevertheless, see supplemental Fig. S7 for a tentative fitting procedure). Based on the fraction of the remaining S2 multiline signal, we estimate this increase to be 2-fold (i.e. ~20%).

The S2 EPR multiline spectra and S2 EPR spectra are known as being exquisitely sensitive to the structure and spin distribution within the cluster (41, 42). On the contrary, the absence of significant differences between these spectroscopic features obtained in the PsbA2-PSII and PsbA3-PSII show that the structure of the Mn$_4$CaO$_5$ is very likely not affected by the PsbA exchange. In addition, the $P_{130}^{450}/P_{180}^{450}$ difference spectra versus the flash number in PsbA2-PSII do not reveal any significant changes in the change distribution over the P$_{130}$P$_{182}$ chlorophyll dimer because the spectra after one, two, or three flashes have a maximum bleaching at 433 nm as in PsbA1-PSII and PsbA3-PSII. Therefore, it seems likely that the increase of the miss
parameter reported above originates from a modification of TyrZ itself. Indeed, several authors have pointed to the electron transfer between TyrZ and P680 as being an important contributor to the miss parameter (for example, see Refs. 56–58 for discussions).

The similar kinetics observed for the S1TyrZ to S2TyrZ transition (τ1/2 ~50 μs), for whichever PsbA protein is involved, is consistent with an unmodified miss parameter on this transition. In the S1TyrZ to S0TyrZ transition, the decay of the Δl/Δ at 292 nm is biphasic (Fig. 3). The fast phase (τ1/2 ~100 μs) seen as a lag phase at 292 nm has been interpreted as reflecting the electrostatically triggered expulsion of one proton from the catalytic center caused by the positive charge near/on TyrZ (59).

The slow phase, attested by an absorption decay with τ1/2 ~1 ms, corresponds to the formation of S0 and O2 and to an additional proton release (Refs. 59–63), and see also Ref. 64 for a recent work dealing with these two phases in *T. elongatus*). From data in Fig. 3, neither the lag phase S3TyrZ to (S3TyrZ)’ nor the (S3TyrZ)’ to S0TyrZ transition seems affected by the PsbA exchange.

For the S3TyrZ to S2TyrZ transition, in which EPR measurements clearly indicate an increase of the miss parameter, the measurements at 292 nm are unfortunately not very informative. Thus, to probe further the TyrZ environment, we measured the reduction of P680i. As previously observed by many groups (see below), this kinetics has multiple components. The fast ones have been interpreted as being kinetically limited by the electron transfer process and were similar in rates in the various samples. The phases developing in the μs to tens of μs time range have been interpreted as being kinetically limited by proton transfer, and these were markedly affected by the PsbA3 to PsbA2 exchange, in particular after the second and third flashes in the series. These components were slower, and their amplitudes were larger in PsbA2-PSII. This, together with the modified split EPR signal detected upon NIR illumination in PsbA2-PSII, shows that TyrZ is indeed the cofactor with modified properties in PsbA2-PSII. Before discussing these structural issues, we would like to note as a side result of the time-resolved TyrZ spectrum in the (S3TyrZ)’ state are also reminiscent of that observed in the TyrZ’ spectrum in the D2-H189L mutant that disrupts the H-bond in which TyrZ is involved (47). Altogether these data thus point to a weaker H-bond between TyrZ and H190.

The data above indicate that the main modifications on the electron donor side of PsbA2-PSII occur at the level of TyrZ. This could be a consequence of the C144P and P173M exchanges, which in turn would modify the H-bond between TyrZ and H190. It is indeed widely agreed that the proton-coupled electron transfer rates for the Tyr oxidation depend on the properties of the *Tyr-O=H=N-His* bonding (e.g. Refs. 26, 31, and 66). It has indeed been shown that, in model compounds, the proton-coupled electron transfer rate from a tyrosine to an oxidant was strongly dependent on the intramolecular distance between the tyrosine and the base that accepts the proton (e.g. Ref. 67). Interestingly, the hydrogen bond between the phenol group of TyrZ and the Ne of H190 in PSII is very short (2.46 Å in Ref. 3). Recently, the rationale underlying this short H-bond has been investigated by a quantum mechanical-molecular mechanical approach (68), and the cluster of four water molecules involved in the Mn4CaO5-TyrZ motif has been shown to play an important role in the stabilization of such a short distance. In this framework, it would not be surprising that a small distortion of such a delicate scaffold would have important consequences on the oxidation of TyrZ by P680i and in particular on those steps that are kinetically limited by the proton transfer within the H-bond network in which TyrZ and His-190 are involved.

In contrast to the marked kinetic effects that we observed on the slow components of the oxidation of TyrZ by P680i, the reduction rates of the various SiTyrZ states were unaffected by the PsbA3 to PsbA2 exchange. As regards the S1 to S2 transition, this is expected because it is only accompanied by substochiometric proton release (69–71). However, the subsequent S2 to S3 and S3 to S0 electron transfer steps are chemically coupled to proton release (e.g. Refs. 72 and 73) and might be affected by the changes in the H-bond network around TyrZ and His-190 discussed above. To our knowledge, the present study is the only one reporting a slowdown of the μs components in the oxidation of TyrZ besides, of course, the H/D experiments (43, 63, 74). Interestingly, kinetic isotope effects have also been reported for the following electron transfer step, *i.e.* the reduction of TyrZ’, at least in the presence of the S2 and S3 states. Notably, the most pronounced kinetic isotope effect has been reported to occur during the S1TyrZ to (S1TyrZ)’ (63), which is assigned to an electrostatically triggered proton release (59, 63). The present observation that the PsbA3 to PsbA2 exchange affects the μs components in the oxidation of TyrZ while keeping unaffected the proton release associated with the S3TyrZ to (S3TyrZ)’ transition suggests that this particular proton release does not originate from the same H-bond network as the one involved in the proton transfer triggered by the formation of TyrZ’. The latter has been described as a sequence of push-pull steps that would be initiated by the transfer of the spectrum measured in the (S3TyrZ)’ state. An up-shift of the gσ value is indicative of a less positive electrostatic environment for TyrZ’ (47). The changes in the hyperfine structure of the time-resolved TyrZ’ spectrum in the (S3TyrZ)’ state are also reminiscent of that observed in the TyrZ’ spectrum in the D2-H189L mutant that disrupts the H-bond in which TyrZ is involved (47). Altogether these data thus point to a weaker H-bond between TyrZ and H190.
phenolic proton from TyrZ to Ne of H190. The identity of the “proton releaser” during the S3 TyrZ− to (S3 TyrZ)+ is not known, and several candidates have been considered. A substrate water molecule is an obvious one (for example, see Ref. 76 for a model in which both the proton and the electron originate from the substrate water molecule). Alternatively, it could be a protonated base, proposed to be CP43-R357 (77), that would undergo a pHk shift upon the formation of the S3 TyrZ− ·HNe(H190)+ state and would, by acting as a proton acceptor from water, promote water splitting. These different proton transfer events thus have essentially different mechanistic implications. Although one mainly reflects electrostatic relaxation, the other sets the stage for all the players in the water-splitting process. In such a framework, it is not surprising that they involve different molecular actors, and the present results support this expectation. Notably, they also point to a necessary conformational change to account for the fact that a new proton releaser that had stayed inactive until the formation of S3 would come into play when S3 TyrZ− ·HNe(H190)+ is formed (for example, see Refs. 73 and 75 for experimental evidences of structural changes in the S state cycle).

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