Environment of TyrZ in Photosystem II from Thermosynechococcus elongatus in which PsbA2 Is the D1 Protein

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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 TyrZ is different from that in PsbA1-PSII and PsbA3-PSII.

Significance: The environment of the cofactors is involved in the tuning of the electron transfer efficiency.

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 TyrZ in the (S2TyrZ)− state is modified; (ii) the split EPR signal arising from TyrZ in the (S1TyrZ)− state induced by near-infrared illumination at 4.2 K of the S3TyrZ state is significantly modified; and (iii) the slow phases of P680+N− reduction by TyrZ are slowed down from the hundreds of μs time resolution to the ms time range, whereas both the S1TyrZ −→ S2TyrZ and the S1TyrZ −→ S0TyrZ + O2 transition kinetics remains similar to those in PsbA(1/3)-PSII.

This result shows that the geometry of the TyrZ phenol and its environment, likely the Tyr-O−−→−Ne−His bonding, are modified in PsbA2-PSII when compared with PsbA(1/3)-PSII. They also point to the dynamics of the proton-coupled electron transfer processes associated with the oxidation of TyrZ being affected. From sequence comparison, we propose that the C144P and P173M substitutions in PsbA2-PSII versus PsbA(1/3)-PSII, respectively located upstream of the α-helix bearing TyrZ and between the two α-helices bearing TyrZ and its hydrogen-bonded partner, His-190, are responsible for these changes.

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Light-driven water oxidation catalyzed by photosystem II (PSII) is the first step in the photosynthetic production of biomass, fossil fuels, and O2 on earth. Refined three-dimensional x-ray structures from 3.5 to 2.9 Å resolution have been obtained using PSII isolated from the thermophilic cyanobacterium Thermosynechococcus elongatus (1, 2). More recently, an x-ray structure at 1.9 Å resolution has been obtained using PSII isolated from the thermophilic cyanobacterium Thermosynechococcus vulcanus (3). PSII core complex is made up of 17 transmembrane protein subunits and 3 extrinsic proteins. In total, it contains 35 chlorophylls, 2 pheophytins, 2 hemes, 1 non heme iron, 2 quinones, 3–4 calcium ions, one of which is part of the Mn4CaO5 cluster, 3 chloride ions, two of which are at 7 Å from the Mn4CaO5 cluster, 11–12 carotenoid molecules, more than 20 lipids, and over 1300 water molecules (3).

The main cofactors involved in the function of PSII are integral components of the D1 and D2 proteins. The exciton resulting from the absorption of a photon is transferred to the photocatalytic trap, which undergoes a charge separation. The positive charge is then stabilized on P680+ and weakly coupled chlorophyll dimer (P D1 and P D2). Then, P680+ oxidizes a tyrosine residue of the D1 polypeptide, TyrZ, which in turn oxidizes the Mn4CaO5 cluster. On the acceptor side, the pheophytin anion (PheoD1) transfers the electron to the primary quinone electron acceptor, Q A, which in turn reduces the second quinone, Q B. Q A is tightly bound and acts as a one-electron carrier, whereas Q B acts as a two-electron and two-proton acceptor with a stable semiquinone intermediate, Q B−. Although the Q B− semiquinone state is tightly bound, the quinone and quinol forms are exchangeable with the quinone pool in the thylakoid membrane (e.g. Refs. 4–9).

3 The abbreviations used are: PSII, photosystem II; Chl, chlorophyll; PPBQ, phenyl-p-benzoquinone; P680+ chlorophyll dimer acting as the second electron donor; Q A, primary quinone acceptor; Q B, secondary quinone acceptor; PheoD1, pheophytin P D1 and P D2; NIR, near-infrared; P D1 and P D2, Chl monomers of P680 on the D1 and D2 side, respectively.

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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 Sₙ state, two molecules of water are rapidly oxidized, the S₀ 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 the FeIII/FeII couple (19). The temperature dependences of the S₂QA state of the FeIII/FeII couple was likely higher in PsbA1-PSII than in PsbA3-PSII (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 ΔpsbA, ΔpsbA₂, T. elongatus deletion mutant (WT*3) from a T. elongatus 43-H strain that had a His₄ tag on the C terminus of CP43 (32) has been previously described in Ref. 33. For making the ΔpsbA, ΔpsbA₂, 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 pB2psbA₂. Then, the psbA₂ gene was substituted with a spectinomycin/streptomycin resistance gene cassette (~2100 bp) by using the plasmid pBΔpsbA₂. For construction of pBΔpsbA₂, 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 pBΔpsbA₃, a DNA fragment of ~900 bp of the 3’-flanking region of the psbA₃ gene (tlr1477) was cloned from T. elongatus wild-type genomic DNA by PCR amplification and subcloned into a plasmid vector pBluescript II SK+ at 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 psbA₃ was ligated to the subcloned plasmid vector at SphI 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’-GCTGTACTTGCCCATCGCTGGGCACCACCTGC-3’) and P2 primer (5’-GGACCTTATCACTTATCAGAGAGT-3’) for psbA₁–psbA₂ region, and using the P3 primer (5’-GGTTGGATCCCGCGGACG-3’) and P4 primer (5’-CCCA-
TGCCCGCAAAACAGC-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 mJ, 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 S₁ 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 CO₂/ethanol bath or illuminated by one or two additional flashes to generate the S₂ and S₃ 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-β-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 TyrZ 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 × 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 CaCl₂, 15 mM MgCl₂, 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 actinic 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 PSII preparations.

RESULTS

The oxygen evolution activity of purified PsbA2-PSII was 3000–3500 μmol of O₂ (mg of Chl)⁻¹ ml⁻¹ h⁻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 TyrZ 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 S₁/S₀ 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 S₁TyrZ → S₀TyrZ, S₂TyrZ → S₁TyrZ and S₃TyrZ transitions in both the PsbA2-PSII and the PsbA3-PSII. At 292 nm, the absorption changes associated with the S₂TyrZ → S₁TyrZ 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 $S_1$, $S_2$, and $S_3$ transitions in PsbA2-PSII ($\text{squares}$) when compared with PsbA3-PSII ($\text{circles}$). Thus, the larger miss parameter in PsbA2-PSII does not originate from a longer lifetime of the $S_i$ states. In addition, we note that the light-induced absorption changes associated with the formation of the $S_2$ state were similar in PsbA2-PSII and PsbA3-PSII. This suggests a similar efficiency for the $S_1$ to $S_2$ transition. We aimed at verifying this conclusion by EPR spectroscopy that provides an exquisitely specific spectrum for the $S_i$ states ($i = 0, 1, 2, 3$).

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 ($\text{circles}$ and $\text{continuous lines}$) or PsbA2-PSII ($\text{squares}$ and $\text{dashed lines}$). Other experimental conditions were similar to those in Fig. 2.

Fig. 4A shows the difference EPR spectra “after-minus-before” flash illumination in PsbA3-PSII ($\text{spectrum a}$) and PsbA2-PSII ($\text{spectrum b}$). After one flash, the characteristic $S_2$ multiline signal centered at $g \approx 2$ and arising from the $\text{Mn}_4\text{CaO}_5$ cluster in the $\text{Mn}^{IV}_{\text{III}}$ redox state with a spin state $S = 1/2$ (see Ref. 41 and references therein for a recent discussion) had an identical shape and a similar amplitude in PsbA3-PSII and PsbA2-PSII. In contrast, after two flashes (Fig. 4B), the amplitude of the $S_2$ multiline signal reflecting the fraction of water-oxidizing complex that did not undergo the $S_2 \rightarrow S_3$ transition was larger in PsbA2-PSII ($\text{spectrum b}$) than in PsbA3-PSII ($\text{spectrum a}$). The amplitude of the positive feature seen between 0 and 1000 G assigned to the spin $S_0$ transition ($2$) was smaller in PsbA2-PSII than in PsbA3-PSII. The temperature used ($8.5 \text{ K}$) was not ideal to measure the $S_3$ EPR signal ($42$) was smaller in PsbA2-PSII than in PsbA3-PSII. The temperature ($8.5 \text{ K}$) was not ideal to measure the $S_3$ EPR signal. Other experimental conditions were similar to those in Fig. 2.

We conclude from the above data that the larger miss parameter is not due to a longer lifetime of any of the $S_i$ states that would result in a larger charge recombination probability. This makes the electron transfer step between $P_{680}^{\cdot}$ and $\text{TyrZ}$ 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, $\text{TyrZ}$, and $\text{QA}$, could potentially contribute to the absorption changes. The most prominent ones, however, are those associated with the formation of $P_{680}^{\cdot}$. The $P_{680}^{\cdot}/P_{680}$ difference spectrum is characterized by a strong Soret band bleaching. After one flash, i.e. in the $S_1P_{680}^{\cdot}$ state, the maximum of the bleaching was observed at 433 nm. After the second flash and third flash, i.e. in the $S_2P_{680}^{\cdot}$ state and $S_3P_{680}^{\cdot}$ 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 $S_1P_{680}^{\cdot}$ state. The red-shift was reversed after the fourth and fifth flashes, i.e. in the $S_0P_{680}^{\cdot}$ and $S_1P_{680}^{\cdot}$ states. This period four oscillation pattern in the $P_{680}^{\cdot}/P_{680}$ spectrum likely originates from an electrostatic effect on the $P_{D1}^{\cdot}P_{D2}^{\cdot}\leftrightarrow P_{D1}'P_{D2}'^{\cdot}P_{D1}'P_{D2}'^{\cdot}$ equilibrium due to the charge(s) stored on/around the $\text{Mn}_4\text{CaO}_5$ 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 $\text{PD1}$ and $\text{PD2}$ chlorophylls is similar in both cases.
FIGURE 5. A, difference spectra around 430 nm. The flash-induced absorption changes were measured at 20 ns in PsbA2-PSII after the first five flashes given on dark-adapted PsbA2-PSII (first flash, black; second flash, blue; third flash, red; fourth flash, green; fifth flash, orange). [Chl] = 25 μg ml−1. B, kinetics of P680+ 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.

Fig. 5B shows the decay of P680+ 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 μs phases were found comparable in PsbA3-PSII and PsbA2-PSII in terms of amplitude and τs. After the second flash (blue symbols) and the third flash (red symbols), the P680+ decay was much slower in PsbA2-PSII, particularly in the hundreds of μs 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 P680+ 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 P680+Q−A+ charge recombination occurred, at least with Qb in the oxidized state. In addition, the presence of PPBQ prevents the formation of stable Qb+ so that the acceptor side is in the same redox state after each flash in the series. Therefore, it is very unlikely that the slower P680+ reduction detected on the second and following flashes originates from a more efficient charge recombination in PsbA2-PSII.

According to the current understanding of the multiphasicity of the reduction of P680+, the ns components are kinetically limited by the electron transfer process, whereas the μs 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 substitu-
tions 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 S3 state, NIR illumination at ~4 K results in the formation of a split EPR signal (48, 49), attributed to a (S2TyrZ)’ 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 (S2TyrZ)’ at the expense of the S3TyrZ state (50). This split signal is attributed to the magnetic interaction between TyrZ, with a spin state S = 1/2 and the Mn4CaO5 cluster possibly in a S = 7/2 spin state (51), and as such is very sensitive to the geometry of the TyrZ/Mn4CaO5 ensemble. As an example of this sensitivity, the split EPR spectrum is significantly modified upon the Ca2+/Sr2+ exchange (52). Importantly, these modifications can be reliably ascribed to changes in the geometry of the bridge between TyrZ and Ca2+/Sr2+ via a water molecule (3) rather than to the alteration of the Mn4CaO5 magnetic structure, which has been independently shown to be only slightly affected by the Ca2+/Sr2+ 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-PSII 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 Mn4CaO5 cluster, at least in the S2 and S3 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 Mn4CaO5 cluster. The EPR properties of TyrZ that may be modified are: (i) the values and localization of
FIGURE 7. A, formation and decay of the Tyr\textsuperscript{Z} signal following laser flash illumination of manganese-depleted PsbA3-PSII measured at 32 magnetic field positions spread over 50 G from 3486 to 3536 G. 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 was obtained by increasing the magnetic field, and the other half was obtained by decreasing the magnetic field. Other instrument settings; modulation amplitude, 4 G; microwave power, 2 milliwatt; microwave frequency, 9.7 GHz; modulation frequency, 100 kHz; and temperature, 293 K. The chlorophyll concentration was 1.1 mg ml\textsuperscript{-1}. B, \textordnary{}{Tyr\textsuperscript{Z} (black)} and \textordnary{}{Tyr\textsuperscript{Z} (red)} spectra extracted from the two-dimensional spectrum in panel A. The \textordnary{}{Tyr\textsubscript{D}} spectrum is the envelope of the baseline before the flash, and the \textordnary{}{Tyr\textsuperscript{Z} spectrum was obtained by extracting the first slice after the flash (i.e., \textsim{}1 ms) after subtraction of the baseline before the flash, which corresponds to the \textordnary{}{Tyr\textsubscript{D}} spectrum.

the spin densities on the carbons and oxygen bearing this spin density; (ii) the orientation of the \textbeta{}-methylene group versus the plan of the phenol ring; (iii) the \textg{\textsubscript{xx}}, \textg{\textsubscript{yy}}, and \textg{\textsubscript{zz}} values; (iv) the relative orientation of \textordnary{}{Tyr\textsuperscript{Z} versus the Mn\textsubscript{4}CaO\textsubscript{5} cluster; and (v) the distance between \textordnary{}{Tyr\textsuperscript{Z} and the Mn\textsubscript{4}CaO\textsubscript{5} cluster. The changes in the magnetic interaction between \textordnary{}{Tyr\textsuperscript{Z} and the Mn\textsubscript{4}CaO\textsubscript{5} cluster could be assessed by a theoretical approach. Nevertheless, although some of the spin signals, in acetate-treated PSII or in the \textsc{S}_1Tyr\textsuperscript{Z} generated at 4 K, have been successfully simulated (53, 54), the magnetic properties of the Mn\textsubscript{4} moiety are poorly understood in the \textsc{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 \textordnary{}{Tyr\textsuperscript{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 \textsc{S}_3Tyr\textsuperscript{Z} in WT \textit{T. elongatus} PSI\textsc{II} (t\textsubscript{1/2} \textsim{}1 ms). To circumvent this limitation, Ca\textsuperscript{2+} and Cl\textsuperscript{−} were substituted by Sr\textsuperscript{2+} and Br\textsuperscript{−} in PsbA3-PSII because it has been shown that this markedly increases the lifetime of \textsc{S}_3Tyr\textsuperscript{Z} (39). In such conditions, the \textsc{S}_3Tyr\textsuperscript{Z} to \textsc{S}_0Tyr\textsuperscript{Z} transition occurs with a t\textsubscript{1/2} close to 7 ms (39), i.e. in a time domain compatible with the reliable detection of \textordnary{}{Tyr\textsuperscript{Z} decay with our EPR set-up. Because in separate experiments (not shown) we checked that the Cl\textsuperscript{−}/Br\textsuperscript{−} exchange had no effect on the \textordnary{}{Tyr\textsuperscript{Z} spectrum and because the Ca\textsuperscript{2+}/Sr\textsuperscript{2+} exchange alone proved sufficient to allow us the full detection of the \textordnary{}{Tyr\textsuperscript{Z} signal, the formation and decay of the \textordnary{}{Tyr\textsuperscript{Z} signals following laser flash illumination were done in Sr\textsuperscript{2+}-containing PsbA2-PSII. These measurements were done at 32 magnetic field positions spread over 50 G and centered on the \textordnary{}{Tyr\textsuperscript{Z} EPR spectrum.

To validate the approach, we first applied the method to manganese-depleted PSII in which the lifetime of \textordnary{}{Tyr\textsuperscript{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 (\textordnary{}{black spectrum}), before the flash, corresponds to the \textordnary{}{Tyr\textsubscript{D}} spectrum, and the second one (\textordnary{}{red spectrum}), immediately after the flash and after subtraction of the baseline for each magnetic field value, corresponds to the \textordnary{}{Tyr\textsuperscript{Z} spectrum. Although the magnetic field resolution is here limited to 50/31 G, the \textordnary{}{Tyr\textsubscript{D} and \textordnary{}{Tyr\textsuperscript{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 \textordnary{}{Tyr\textsuperscript{Z} signal was kinetically detectable only in the
S$_t$ T$_{yrZ}^*$ to S$_0$ T$_{yrZ}$ transition. In addition, because 16 consecutive flashes were averaged for each sample, the amplitude of the T$_{yrZ}^*$ signal should be at most one-fourth of that detected in manganese-depleted PSII in which almost one T$_{yrZ}^*$ species is detectable upon each flash. Fig. 8B shows the T$_{yrZ}^*$ spectrum recorded in manganese-depleted PSII in Fig. 7 (black spectrum) and the T$_{yrZ}^*$ spectrum recorded immediately after the flash in Sr/Br-containing PsbA3-PSII (red spectrum, solid line). The latter has been multiplied 4-fold (dashed red line) to ease its comparison with the manganese-depleted case. Within experimental accuracy, the T$_{yrZ}^*$ spectrum in the (S$_t$ T$_{yrZ}^*$)’ state was found identical to the T$_{yrZ}^*$ spectrum in manganese-depleted PSII. In addition, we did not find any significant changes in the shape of the T$_{yrZ}^*$ spectrum occurring while it decays (see supplemental Figs. S3 and S4).

Finally, the two-dimensional EPR experiment was performed in Sr-containing PsbA2-PSII. Fig. 9 compares the T$_{yrZ}^*$ spectra detected in PsbA3-PSII (black spectrum) and that recorded in PsbA2-PSII (red spectrum) and evidences small but significant differences between the PsbA2-PSII and PsbA3-PSII T$_{yrZ}^*$ spectra in the (S$_t$ T$_{yrZ}^*$)’ states.

**DISCUSSION**

In this study, we describe for the first time the construction of a *T. elongatus* mutant in which both the psbA$_1$ and the psbA$_3$ genes have been deleted, so thus the mutant expresses only the psbA$_2$ gene. The O$_2$-evolving activity of PSII purified from this strain is close to that measured with PsbA1-PSII (3,900 μmol of O$_2$ (mg of Chl)$^{-1}$ h$^{-1}$) and approximately half of that found for PsbA3-PSII (6,000 μmol of O$_2$ (mg of Chl)$^{-1}$ h$^{-1}$). In the current understanding, the limiting step of the overall oxygen evolution in vitro is the exchange of the doubly reduced Q$_B$H$_2$ molecule by an oxidized one. Thus, these different oxygen-evolving activities may stem from amino acid substitutions located close to the quinone binding sites. For example, in the x-ray three-dimensional structure of PsbA1-PSII (3), S270 and C212 locate close to the QB binding site (supplemental Fig. S1). S270 is replaced by an alanine in both PsbA2-PSII and PsbA3-PSII, and C212 is replaced by an alanine in PsbA2-PSII and a serine in PsbA3-PSII. However, sequence alignment does not permit the identification of other amino acids that would account for, on the one hand, the similarity between PsbA1-PSI and PsbA2-PSII and, on the other hand, the dissimilarities between these two and PsbA3-PSII. Probably, several amino acid substitutions between PsbA1-PSII, PsbA2-PSII, and PsbA3-PSII could potentially induce long distance effects acting on the hydrogen bond network, the water molecule network, the binding of lipids, and possibly the fine tuning of the interactions between the D1/D2 α-helices.

The C144P and P173M exchanges are two of the less conservative differences between PsbA2 and PsbA1(1/3). In PsbA1 and PsbA3, the C144 is located in helix C on the periplasmic side just before the α-helix bearing T$_{yrZ}$ (the Y161), and the P173 is located in the loop between the C and D helices bearing T$_{yrZ}$ 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 T$_{yrZ}$ (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 T$_{yrZ}$ and H190 is expected to have significant structural consequences on the local environment of T$_{yrZ}$ 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 T$_{yrZ}$ 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 S$_2$ formation estimated from the amplitude of the S$_2$ EPR multiline signal was identical in PsbA2-PSII and PsbA3-PSII, thus showing that the yield of the S$_1$ to S$_2$ transition is not significantly affected (Fig. 4). In contrast, the smaller amplitude of the absorption change detected upon the second flash and the smaller S$_3$ EPR signal and the larger fraction of remaining S$_2$ multiline signal detected after two flashes applied to PSII centers synchronized in the S$_1$ state prior to the flash illumination suggest that in PsbA2-PSII, the miss parameter strongly increases in the S$_2$ to S$_3$ 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 S$_2$ multiline signal, we estimate this increase to be 2-fold (i.e. ~20%).

The S$_2$ EPR multiline spectra and S$_3$ 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 $P_173^{173}/P_{980}$ difference spectra versus the flash number in PsbA2-PSII do not reveal any significant changes in the charge distribution over the P$_{137}$P$_{132}$ 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 S1, TyrZ to S1, TyrZ transition (τs ~ 50 μs), for whichever PsbA protein is involved, is consistent with an unmodified miss parameter on this transition. In the S1, TyrZ to S0, TyrZ transition, the decay of the ΔI/I at 292 nm is biphasic (Fig. 3). The fast phase (τf ~ 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 τs ~ 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 this two phases in T. elongatus). From data in Fig. 3, neither the lag phase S1, TyrZ to (S1, TyrZ)′ nor the (S3, TyrZ)′ to S0TyrZ transition seems affected by the PsbA exchange.

For the S2, TyrZ to S2, TyrZ 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 P680+. 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 that as a side result of the present study, we report here the first TyrZ EPR spectrum in the (S1, TyrZ)′ state in active PSII. We show that it is similar to that in manganese-depleted PSII. From W-band EPR, the geometry of TyrZ in manganese-depleted PSII crystals was found to be similar to that expected from the geometry of TyrZ in the dark-adapted state of Mn4CaO5-containing crystal (65). Thus, the geometry of TyrZ in the (S1, TyrZ)′ state is also likely similar to that of TyrZ in the S1 state.

Several structural reasons may be considered to account for the different properties of TyrZ. One is a change in the orientation of the TyrZ radical with respect to the Mn4CaO5 cluster that would modify the magnetic interaction between TyrZ and the Mn4CaO5 cluster. To further assess this, we attempted to characterize the g-tensor of TyrZ by high field EPR. We note, however, that this study was performed with manganese-depleted PsbA2-PSII (supplemental Fig. S5), so its conclusions cannot be straightforwardly extrapolated to oxygen-evolving PSII. Although small, the changes indicate without ambiguities that the g-resonance of the TyrZ spectrum was broader and likely up-shifted. This could explain the modified X-band EPR spectrum measured in the (S3, TyrZ)′ state. An up-shift of the gx 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 (S3, TyrZ)′ 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 P680+ 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 P680+, the reduction rates of the various S, TyrZ states were unaffected by the PsbA3 to PsbA2 exchange. As regards the S2 to S3 transition, this is expected because it is only accompanied by substochiometric proton release (69–71). However, the subsequent S3 to S4 and S4 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 S1, TyrZ to (S1, TyrZ)′ (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 S3, TyrZ to (S3, TyrZ)′ 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
phenolic proton from Tyr$_Z$ to Ne of H190. The identity of the “proton releaser” during the S$_3$Tyr$_Z$ to (S$_3$Tyr$_Z$)$_3$’ 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 pK$_A$ shift upon the formation of the S$_3$Tyr$_Z$⋯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 S$_3$ would come into play when S$_3$Tyr$_Z$⋯HNe(H190)$^+$ is formed (for example, see Refs. 73 and 75 for experimental evidence of structural changes in the S$_3$ state cycle).

REFERENCES


