

Evidence that D1-His332 in Photosystem II from *Thermosynechococcus elongatus* Interacts with the S₃-State and not with the S₂-State[†]

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Received June 24, 2009; Revised Manuscript Received July 22, 2009

ABSTRACT: Oxygen evolution by Photosystem II (PSII) is catalyzed by a Mn₄Ca cluster. Thus far, from the crystallographic three-dimensional (3D) structures, seven amino acid residues have been identified as possible ligands of the Mn₄Ca cluster. Among them, there is only one histidine, His332, which belongs to the D1 polypeptide. The relationships of the D1-His332 amino acid with kinetics and thermodynamic properties of the Mn₄Ca cluster in the S₂- and S₃-states of the catalytic cycle were investigated in purified PSII from *Thermosynechococcus elongatus*. This was done by examining site-directed D1-His332Gln and D1-His332Ser mutants by a variety of spectroscopic techniques such as time-resolved UV–visible absorption change spectroscopy, cw- and pulse-EPR, thermoluminescence, and measurement of substrate water exchange. Both mutants grew photo-autotrophically and active PSII could be purified. On the basis of the parameters assessed in this work, the D1-His332(Gln, Ser) mutations had no effect in the S₂-state. Electron spin–echo envelope modulation (ESEEM) spectroscopy also showed that possible interactions between the nuclear spin of the nitrogen(s) of D1-His332 with the electronic spin $S = 1/2$ of the Mn₄Ca cluster in the S₂-state were not detectable and that the D1-His332Ser mutation did not affect the detected hyperfine couplings. In contrast, the following changes were observed in the S₃-state of the D1-His332 mutants: (1) The redox potential of the S₃/S₂ couple was slightly increased by ≤ 20 meV, (2) The S₃-EPR spectrum was slightly modified, (3) The D1-His332Gln mutation resulted in a ~ 3 fold decrease of the slow (tightly bound) exchange rate and a ~ 2 fold increase of the fast exchange rate of the water substrate molecules. All these results suggest that the D1-His332 would be more involved in S₃ than in S₂. This could be one element of the conformational changes put forward in the S₂ to S₃ transition.

Light-driven water oxidation by the Photosystem II (PSII)¹ enzyme is responsible for the O₂ on Earth and is at the origin of the production of most of the biomass. Refined three-dimensional (3D) X-ray structures from 3.5 Å to 2.9 Å resolution have been obtained using PSII isolated from the thermophilic cyanobacterium *Thermosynechococcus elongatus* (1–3). PSII is made up of 20 membrane protein subunits, 35 chlorophyll molecules, 2 pheophytin molecules at least 12 carotenoid molecules, ≥ 25

lipids, 2 hemes, 1 nonheme iron, 2(+1) quinones, 4 Mn ions, 1 Ca²⁺ ion, and at least 1 Cl[−] ion (1–5).

Absorption of a photon by a chlorophyll is followed by the formation of a radical pair in which the pheophytin molecule, Pheo_{D1}, is reduced and the accessory chlorophyll molecule, Chl_{D1}, is oxidized (6–8). The cation is then quickly transferred and stabilized on P₆₈₀, a weakly coupled chlorophyll dimer; see, for example, refs 9 and 10 for energetic considerations. The pheophytin anion transfers its unpaired electron to a quinone, Q_A, which in turn reduces a second quinone, Q_B. P₆₈₀^{•+} oxidizes a tyrosine residue of the D1 polypeptide, Tyr_Z, which in turn oxidizes the Mn₄Ca-cluster.

The Mn₄Ca-cluster acts as an oxidizing-equivalent accumulating device as well as the active site for water oxidation. During the enzyme cycle, the catalytic site goes through five sequential redox states, denoted S_{*n*} where *n* varies from 0 to 4 upon the absorption of four photons (11). Upon formation of the S₄ state two molecules of water are rapidly oxidized, the S₀-state is regenerated and O₂ is released.

The mechanism by which water is oxidized and O₂ is produced is still largely unknown, for example, refs 12–19. First, uncertainties still remain as to the structure of the native Mn₄Ca cluster owing to the reduction by the X-ray beam of the native high

[†]This study was supported in part by the JSPS and CNRS under the Japan-France Research Cooperative Program, by the EU/Energy project SOLAR-H2 (contract no. 212508), and by Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Science, Sports, Culture and Technology (18770116 for M.S.).

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¹Abbreviations: PSII, photosystem II; WT*, *T. elongatus* strain with a His-tag on the C terminus at CP43 and in which the *psbA1* and *psbA2* genes are deleted. Chl, chlorophyll; CP43 and CP47, chlorophyll-binding proteins; P₆₈₀, primary electron donor; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; Pheo, pheophytin; DCBQ, 2,6-dichloro-*p*-benzoquinone; PPBQ, phenyl-*p*-benzoquinone, EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; TL, thermoluminescence; MES, 2-(*N*-morpholino) ethanesulfonic acid; Me₂SO, dimethyl sulfoxide; Cm, chloramphenicol; Sp, spectinomycin; Sm, streptomycin; β DM, beta dodecyl maltoside.

valence Mn_4Ca cluster back to the Mn^{II} state (20, 21). Second, the transition from S_3TyrZ^* to S_0 likely involves several reaction intermediates. Until now, these have largely escaped detection for various reasons among which (1) the reduction of S_3TyrZ^* is the limiting step for water oxidation in the native enzyme, and (2) experimental methods for trapping potential intermediate states are still lacking; see ref 22 for a recent elegant thermodynamic approach, the conclusions of which however have been recently challenged (23). For all these reasons, the mechanism of water oxidation still remains uncharacterized. As a consequence, the ligand environment of the Mn_4Ca -cluster and the role of the ligands in the overall mechanism still require being accurately characterized.

According to the structures (1–3), seven amino acids ligating the Mn_4Ca cluster have been identified. Among these, six belong to the D1 protein (D1-Ala344, D1-Glu189, D1-His332, D1-Asp342, D1-Glu333, and D1-Asp170) and one belongs to the CP43 protein (CP43-Glu354). The role of these amino acids in maintaining a functional Mn_4Ca cluster has been investigated by several groups before the 3D structures came out. This has been done by extensive site-directed mutagenesis in the mesophilic cyanobacterium *Synechocystis* 6803, reviewed in ref 15. The confrontation of these studies to the 3D structure rises however an intriguing issue. Indeed, although the S_2 multiline EPR signal has been considered until now as being extremely sensitive to the environment of the Mn_4Ca cluster, mutating most of the side-chains proposed to act as ligand to this cluster, the D1-Ala344 (Gly, Val, Asp, Asn) (24), D1-Glu189Gln (25, 26), D1-Asp342Asn (27), D1-Asp170His (28), and CP43-Glu354Gln (29), had hardly any effect on the S_2 multiline EPR signal within experimental accuracy. In the case of D1-His337, an amino acid residue in the second coordination sphere, no spectroscopic studies have yet been reported on any D1-His337 mutant (15). For the D1-His332Glu mutant, the EPR characteristics were similar to those of a Ca-depleted sample; that is, the S_2 multiline signal was modified and the electron transfer was blocked after S_2TyrZ^* formation (30, 31). Nevertheless, the low quantum yield for the formation of S_2 as well as the lack of the unusual stable S_2 state observed in Ca^{2+} -depleted PSII with these EPR characteristics were taken as arguments by the authors (30) against the release of Ca^{2+} in the D1-His332Glu mutant.

In addition to the lack of modification of the S_2 multiline EPR signal several mutations are without any influence on the S_r -state cycle, at least in a fraction of the centers, such as D1-Glu189(Lys, Arg, Gln) (25, 26), D1-Asp342Asn (27), D1-Asp170His (28). For D1-Ala344, D1-Glu189, D1-Asp342, D1-Glu333, and D1-Asp170, in most cases, the reported decrease in the activity of the mutants followed a decrease in the fraction of manganese containing PSII (24–31) suggesting, as already proposed for D1-Asp170His (28), that these amino acids are more essential to the assembly and/or the repair of PSII than to the oxygen evolution mechanism per se. There are four exceptions for which mutations strongly affect the electron transfer at the donor side. Surprisingly, these are (i) CP43-Arg357 (32), D1-Asp61 (33, 34), and D1-His337 (15) which all belong to the second coordination sphere of the Mn_4Ca cluster according to the 3D structures and (ii) the mutants at the D1-His332 position (15), as discussed above. For the latter, in *Synechocystis* 6803, any of the D1-His332 mutants which have been studied were photoautotrophic and only the D1-His332(Gln, Ser) mutants evolved oxygen in whole cells at 10–15% the rate of wild-type cells (15, 35).

Since PSII isolated from *T. elongatus* is expected to be more robust than that purified from *Synechocystis* 6803, we have performed a study of D1-His332Gln and D1-His332Ser mutants in this thermophilic cyanobacterium with the hope that a high proportion of O_2 evolving PSII centers could be purified. This was indeed the case and we report here a characterization of these two mutants by a variety of spectroscopic techniques such as time-resolved UV–visible absorption change spectroscopy, cw- and pulse-EPR, Thermoluminescence and measurement of substrate water exchange. We found some evidence that the D1-His332(Gln, Ser) mutations affect some properties of the S_3 -state.

EXPERIMENTAL PROCEDURES

Construction of the D1-His332Gln and D1-His332Ser Mutants. Site-directed D1-His332Gln and D1-His332Ser mutations were constructed in the *psbA₃* gene of the thermophilic cyanobacterium *Thermosynechococcus elongatus*. For creating mutations, the plasmid vector pUC19 inserting the *psbA₃* gene (including 790 bp upstream and 870 bp downstream the *psbA₃* open reading frame) and spectinomycin (Sp) and streptomycin (Sm) resistance gene cassette just after the stop codon of *psbA₃* (36) was used. The mutations D1-His332Gln and D1-His332Ser were done by using a Quick-Change XL Site-Directed Mutagenesis kit (Stratagene). When making mutations, *Aat* II and *Bsp* H I recognition sites were created in D1-His332Gln and D1-His332Ser, respectively, around position of His as previously described (36, 37, 39). The DNA sequences are for D1-His332Ser: ...ATGGAAGTCATGAGCGAGCGCAAT... and for D1-His332Gln: ...ATGGACGTCATGCAGGAGCGCAAT... Characters that are in italic are the restriction enzyme recognition sites and characters that are underlined are the D1–332 position. The plasmids bearing mutations were transformed by electroporation into *T. elongatus* WT* cells. WT* was the host strain in which both the *psbA₁* and *psbA₂* genes were deleted (36) and which contains a His₆-tag fused to the C-terminus of CP43 (38). Single colonies were selected on DTN agar plate containing 12.5 μg of Sp $\cdot\text{mL}^{-1}$, 5 μg of Sm $\cdot\text{mL}^{-1}$, 20 μg of Km $\cdot\text{mL}^{-1}$, and 2.5 μg of Cm $\cdot\text{mL}^{-1}$. Segregation of all the *psbA₃* copies in genome of the deletion mutant was confirmed by digestion of *psbA₃* with *Aat* II and *Bsp* H I in D1-His332Gln and D1-His332Ser, respectively, after PCR amplification of the mutated region as described in refs (36–38). The sequences of the genomic DNA in the transformants which were used for this work, after complete segregation of the cells, were confirmed by using a CEQ2000 DNA Analysis System (Beckman).

Purification of Thylakoids and PSII Core Complexes. The transformed cells (and fully segregated) were grown in 1 L cultures of DTN in 3-L Erlenmeyer flasks in a rotary shaker with a CO_2 -enriched atmosphere at 45 °C under continuous light in the presence of the corresponding antibiotics (see above). Thylakoids and PSII core complexes were prepared essentially as described earlier (36, 37, 39) with the following modifications. Glycerol was avoided from the elution step to the end of the purification. Indeed, glycerol was found to prevent the full detection of the S_3 EPR signal (40). The medium used to elute the PSII core complexes bound to the Ni^{2+} resin contained 1 M betaine, 200 mM L-histidine, 100 mM NaCl, 15 mM CaCl_2 , 15 mM MgCl_2 , 40 mM Mes, 0.06% βDM , pH 6.5. After elution, PSII were washed and concentrated by using Amicon Ultra-15 concentrator devices (Millipore) with a 100 kDa cutoff. The washing medium and the final resuspending medium were 1 M betaine, 15 mM CaCl_2 , 15 mM MgCl_2 , 40 mM Mes, pH 6.5

(adjusted with NaOH). Routinely, the total amount of Chl before the breaking of the cells was ≈ 150 – 200 mg, and the yield after PSII purification in terms of Chl amounts was ≈ 3 – 5% . PSII were stored in liquid nitrogen at a concentration of about 2 mg of Chl $\cdot$ mL $^{-1}$. Betaine was used at a concentration of 1 M which has been shown to fully protect the O $_2$ activity in modified PSII and mutants in *T. elongatus* (36, 37, 39–41).

Oxygen Evolution under Continuous Light. Oxygen evolution of PSII under continuous light was measured at 25°C by polarography using a Clark-type oxygen electrode (Hansatech) with saturating white light at a Chl concentration of $5\ \mu\text{g}$ of Chl $\cdot$ mL $^{-1}$ in the media described above. A total of 0.5 mM DCBQ (2,6-dichloro-*p*-benzoquinone, dissolved in dimethyl sulfoxide) was added as an electron acceptor. The betaine, DCBQ, and Q $_B$ react in the minutes time range in the presence of O $_2$ (not shown). For that reason, measurement of PSII activity was done less than 1 min after the addition of DCBQ.

Time Resolved UV–visible Absorption Change Spectroscopy. Absorption changes were measured with a lab-built spectrophotometer (42) where the absorption changes are sampled at discrete times by short flashes. These flashes were provided by a neodymium:yttrium aluminum garnet (Nd:YAG) pumped (355 nm) optical parametric oscillator, which produces monochromatic flashes (1 nm full-width at half-maximum) with a duration of 6 ns. Excitation was provided by a dye laser (685 nm, 1 mJ) pumped by the second harmonic of a Nd:YAG laser. PSII was used at $25\ \mu\text{g}$ of Chl $\cdot$ mL $^{-1}$ in 1 M betaine, 15 mM CaCl $_2$, 15 mM MgCl $_2$, and 40 mM MES (pH 6.5). PSII were dark-adapted for 1 h at room temperature before the additions of either 0.1 mM phenyl-*p*-benzoquinone (PPBQ, dissolved in Me $_2$ SO) or DCMU (dissolved in ethanol).

Thermoluminescence Measurements. PSII core complexes were suspended in 1 M betaine, 15 mM CaCl $_2$, 15 mM MgCl $_2$, and 40 mM MES (pH 6.5), and thermoluminescence (TL) glow curves were measured with a lab-built apparatus (43). The Chl concentration was $0.2\ \mu\text{g}$ of Chl $\cdot$ mL $^{-1}$. PSII were then dark-adapted for 1 h. The samples were illuminated at 5°C by using a saturating xenon flash (SL-230S; Sugawara, Japan) and then rapidly chilled to 77 K with liquid N $_2$. The frozen samples were then heated at a constant rate of $40^\circ\text{C} \cdot \text{min}^{-1}$ and TL emission was detected with a photomultiplier (Hamamatsu, R943–02).

EPR Spectroscopy. cw-EPR spectra were recorded using a standard ER 4102 (Bruker) X-band resonator with a Bruker Elexsys 500 X-band spectrometer equipped with an Oxford Instruments cryostat (ESR 900). Flash illumination at room temperature was provided by a Nd:YAG laser (532 nm, 550 mJ, 8 ns Spectra Physics GCR-230-10). Pulsed EPR experiments were performed with a Bruker ESP580 spectrometer equipped with an Oxford Instruments cryostat. Field-swept and ESEEM spectra were recorded using a 3-pulse sequence $\pi/2$ – τ – $\pi/2$ – T – $\pi/2$ –echo. The duration of the $\pi/2$ pulses was 16 ns. For the ESEEM, 800 points were recorded and T was incremented by 8 ns steps from its initial value equal to 60 ns. Fourier transform of the time domain data was done after: (1) subtraction of a background double exponential function, (2) zero-filling to 1024 points, (3) dead-time reconstruction by simulating peaks in the frequency domain followed by a back Fourier transform to fill the missing data in the dead time, and (4) Fourier transform of the full data (experimental + reconstructed dead time).

PSII samples at 1.1 mg of Chl $\cdot$ mL $^{-1}$ for cw-EPR and ≈ 4 mg of Chl $\cdot$ mL $^{-1}$ for pulse-EPR were loaded in the dark into quartz EPR tubes and further dark-adapted for 1 h at room

temperature. Then, the samples for cw-EPR were synchronized in the S $_1$ state with one preflash (44). After another dark period of 1 h at room temperature, 0.5 mM PPBQ was added (the final concentration of dimethyl sulfoxide was $\approx 2\%$). Then, the samples were frozen in the dark to 198 K and then transferred to 77 K. Prior to the measurements the samples were degassed at 198 K as was already described (39).

Analysis of the data was done by using Excel (Microsoft), Mathcad 14 (Parametric Technology Corporation), and Origin 7.5 (OriginLab Corporation).

Mass Spectrometric Measurements. Frozen PSII core samples were thawed and diluted at $0.125\ \mu\text{mol}$ of Chl $\cdot$ mL $^{-1}$ in the assay buffer containing 40 mM MES (pH 6.5), 15 mM MgCl $_2$, 15 mM CaCl $_2$, 10% glycerol, and 1.2 M betaine with 0.5 mM K $_3$ Fe(CN) $_6$ serving as an electron acceptor. To circumvent prolonged dark adaptation/preflash cycles associated with the S-state lifetimes (45, 46), the samples were loaded under complete darkness with IR goggles. The rapid mixing sample chamber interfaced to the mass spectrometer has been described earlier (47–49), and was temperature controlled to 10°C . Experiments were performed using purified PSII preset into the S $_3$ state and injection (mixing rate $k_{\text{inj}} = 200\ \text{s}^{-1}$) of a $25\ \mu\text{L}$ addition of 95% H $_2$ ^{18}O to obtain the final ^{18}O enrichment of $12.0 \pm 0.5\%$. The timing sequence and analysis are given in ref 49. Analysis of the biphasic plots of $m/z = 34$ exchange were fit to the sum of two exponential functions revealing a fast phase k_2 and slow phase k_1 of H $_2$ ^{18}O exchange.

$$Y_C = 0.57(1 - \exp(-k_2 t)) + 0.43(1 - \exp(k_1 t)) \quad (1)$$

Analysis of the $m/z = 36$ exchange was to a single exponential function arising from the rate determining slow exchange k_1 of H $_2$ ^{18}O exchange.

$$Y_C = 1 - \exp(k_1 t) \quad (2)$$

The two data sets were globally fit with eqs 1 and 2 to derive one set of k_1 parameters.

RESULTS

To study the effects of the D1-His332 mutations on water splitting, we conducted various spectroscopic studies on the Mn $_4$ Ca cluster in PSII. First, we examined O $_2$ evolution under steady-state illumination. The activity of purified WT* PSII was routinely determined as 4500 – $6000\ \mu\text{mol}$ of O $_2$ /mg of Chl $\cdot$ h $^{-1}$, whereas activity in the mutants was somewhat lower at 3800 – $4500\ \mu\text{mol}$ of O $_2$ /mg of Chl $\cdot$ h $^{-1}$. The O $_2$ activity of *T. elongatus* after D1-His332(Gln,Ser) mutations is much greater than in the case of similar mutations in *Synechocystis* PCC 6803 since in these mutant strains the photoautotrophic growth was 10 – 15% of wild type cells (35). In the *T. elongatus*, the activity in purified PSII was decreased by $\sim 20\%$ when compared to WT* PSII.

To gain further insight into integrity and potential turnover limitations of the Mn $_4$ Ca cluster transient absorption spectroscopy was performed at 292 nm to follow the oxidation changes of the Mn ions. The measurements were done at 19°C . Figure 1 shows the S $_n$ -state cycle probed by the amplitude of the flash-induced absorption changes in the WT*, the D1-His332Gln mutant and the D1-His332Ser mutant. Absorption measurements at 292 nm were recorded in the hundred of millisecond time range; that is, after the reduction of Tyr $_Z^*$ by the Mn $_4$ Ca cluster was complete. This wavelength corresponds to an isosbestic point for PPBQ $^-$ /PPBQ (not shown), the added electron acceptor, and is in a spectral region where the absorption of the Mn $_4$ Ca cluster

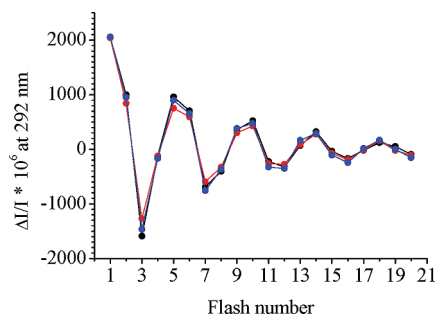


FIGURE 1: Sequence of the amplitude of the absorption changes at 292 nm during a series of saturating flashes spaced 400 ms apart and given on WT*-PSII (black symbols), D1-His332Gln PSII (red symbols), and D1-His332Ser PSII (blue symbols). The samples at 25 μg of Chl/mL were dark-adapted for 1 h at room temperature before the addition of 100 μM PPBQ (dissolved in Me₂SO). The measurements were done 100 ms after each flash.

depends on the S-states (50, 51). The oscillating pattern with a period of four is clearly observed for all three types of PSII preparations in Figure 1. By using the formula developed by Lavorel (52) and the analysis developed by Lavergne (51), the three oscillating patterns shown in Figure 1 were fitted together as previously described (46). Each of the three different differential extinction coefficients, $\Delta\epsilon_0$, $\Delta\epsilon_1$, $\Delta\epsilon_2$, corresponding to the S_0 to S_1 , S_1 to S_2 , and S_2 to S_3 transitions, respectively, were kept fixed for the three types of PSII and they were found equal to 400×10^{-6} , 1500×10^{-6} , and 1200×10^{-6} , respectively, in $\Delta I/I$ unit for the sample concentration (25 μg of Chl/mL) and path length (2.5 mm). In this experiment, a double hit parameter, 4–5%, was used to account for the small actinic effect of the measuring beam. The miss parameter was found to be close to 10% for the three samples. The S_0/S_1 ratio was allowed to vary for each type of sample. It was found to be 0/100 for the WT* PSII and 10/90 for the two mutants (Supporting Information).

The results in Figure 1 show that in purified PSII both the D1-His332Gln and D1-His332Ser mutations neither significantly alter the S_n -state cycle in terms of transition probability, nor increase the fraction of inactive PSII centers based on the absorption at 292 nm per unit of Chl concentration. Therefore, the slightly smaller O_2 evolution activity under continuous illumination in purified PSII does not reflect a lower proportion of active centers in the D1-His332Gln and D1-His332Ser mutants but rather a slight kinetic limitation.

Although the period four oscillation pattern was hardly affected by the mutations, we characterized the kinetics of electron transfer associated with each of the individual steps to gain further insights into the consequences of the mutations. For that we recorded the absorption changes at 292 nm over the microsecond to millisecond time-range after each flashes of a series given to dark-adapted PSII (Figure 2). Absorption changes at this wavelength reflect the Mn_4Ca -cluster valence change and the Tyr_Z redox state change occurring in the $S_1\text{Tyr}_Z^\bullet$ to $S_2\text{Tyr}_Z^\bullet$, $S_2\text{Tyr}_Z^\bullet$ to $S_3\text{Tyr}_Z^\bullet$, $S_3\text{Tyr}_Z^\bullet$ to $S_0\text{Tyr}_Z$ and $S_0\text{Tyr}_Z^\bullet$ to $S_1\text{Tyr}_Z$ transitions (46, 50, 51). As shown in Figure 2, the transient absorption changes hardly differed between the three samples after the first, second, and third flash (and also in subsequent flashes, data not shown). Increasing the temperature up to 53 $^\circ\text{C}$, a more physiological temperature for *T. elongatus*, reduced the miss parameter to $\approx 5\%$ in both the WT* and mutant PSII (not shown). Time-resolved absorption changes measured at 53 $^\circ\text{C}$ and at 292 nm revealed no kinetic differences between the WT* and the D1-His332Gln and D1-His332Ser mutants (not shown).

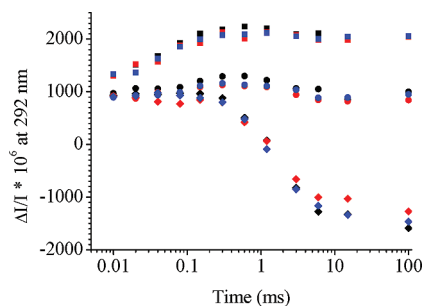


FIGURE 2: Kinetics of the absorption changes at 292 nm after the first flash (squares), the second flash (circles), and the third flash (diamond) given to dark-adapted WT*-PSII (black), D1-His332Gln PSII (red), and D1-His332Ser PSII (blue). Other experimental conditions were similar to those in Figure 1.

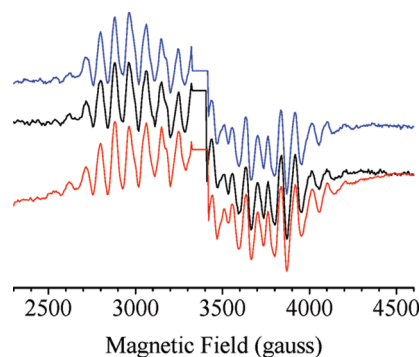


FIGURE 3: Light minus dark EPR spectra induced by one flash at room temperature in the presence of 1 mM PPBQ and recorded on WT*-PSII (black spectrum), D1-His332Gln PSII (red spectrum), and D1-His332Ser PSII (blue spectrum). Sample concentration was 1.1 mg of Chl/mL. A preflash was given to synchronize all the centers in S_1 . Instrument settings: modulation amplitude, 25 gauss; microwave power, 20 mW; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; Temperature, 8.5 K. The central part of the spectra corresponding to the $\text{Tyr}_D^\bullet$ region was deleted.

As mentioned in the introduction, site-directed D1-His332Glu mutation in *Synechocystis* 6803 resulted in the complete inhibition of the S_n -state cycle after $S_2\text{Tyr}_Z^\bullet$ formation as well as in a modified multiline EPR signal in the S_2 state (30, 31). The EPR multiline spectrum arising from the S_2 state is centered at $g \approx 2$ and spread over about 1800 G. It is made up of at least 18 lines, each separated by approximately 80 G, and arises from a spin 1/2 (ground) state, very likely from a magnetic Mn tetramer, $\text{Mn}^{\text{IV}}_3\text{Mn}^{\text{III}}$ (53–56). Since the parameters determining the rate of the $S_n \rightarrow S_{n+1}$ transitions are largely unknown, the possibility that the mutations affect the environment of the Mn_4Ca cluster with little consequences on the rates remains. Because it probes the spin coupling between the Mn ions in the tetramer, EPR spectroscopy is well suited to characterize possible changes in the coordination sphere of the Mn ions. The results in Figure 3 show that in the D1-His332Gln and D1-His332Ser mutants both the amplitudes and shapes of the S_2 -multiline signal recorded after one flash given at room temperature were indistinguishable from that in WT*-PSII.

The resolved hyperfine structure of the S_2 multiline EPR spectrum originates from the interaction between the electronic and the nuclear spins of the four Mn ions. To obtain information on the interactions between the electronic spin and the surrounding nuclei of the solvent and/or the protein matrix a technique allowing the resolution of the weak hyperfine interactions is required. ESEEM spectroscopy is suited for doing so, and

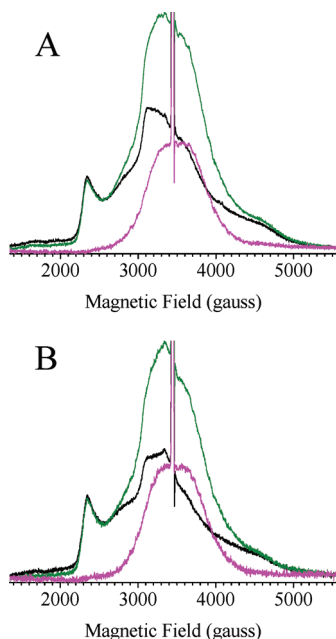


FIGURE 4: Field-swept echo spectra in WT* (A) and D1-His332Ser (B). The amplitude of the spectra was normalized to the same PSII concentration by using the Tyr_D⁺ signal as a reference. Spectra were recorded in the dark-adapted state (black spectra) and after illumination at 200 K (green spectra). The magenta spectra are the light *minus* dark spectra. The echo resulted from a three-pulse sequence $\pi/2-\tau-\pi/2-T-\pi/2$ -echo. The spectra shown are the sum of spectra recorded for $T = 60$ ns and τ values equal to 136, 168, 200, and 232 ns. Temperature, 4.2 K; microwave frequency, 9.7 GHz. Shot repetition time = 8 ms. The central part of the spectra corresponding to the Tyr_D⁺ region was deleted.

the results of such an approach are shown in Figure 4 for the WT* PSII and the D1-His332Ser mutant. The D1-His332Gln mutant was not used here since this substitution maintains a nitrogen in the possibly interacting amino acid side-chain.

Figure 4 shows the field-swept spectra in WT* PSII (panel A) and D1-His332Ser PSII (panel B). Spectra were recorded in the dark-adapted samples (black spectra) and after illumination at 200 K (green spectra), that is, in the S_2 -state. The magenta spectra show the difference between the spectra obtained after illumination and in the dark. The spectra recorded in the dark-adapted samples, that is, in the S_1 -state, arise from the fully oxidized Cyt_c₅₅₀ and possibly from a low amount of oxidized Cyt_b₅₅₉ (57, 58). The light-induced field-swept spectra correspond to the S_2 -multiline signal. As for the cw-EPR experiment, Figure 4 shows that the S_2 signal induced by 200 K illumination had a similar amplitude in the WT* PSII and D1-His332Ser PSII samples.

To disentangle the various species which may contribute to the ESEEM spectra, these were recorded in S_1 and S_2 at nine magnetic field values from 2340 to 3940 gauss by 200 gauss steps and with τ values equal to 136, 168, 200, and 232 ns. At 2340 gauss, only the cytochrome(s) contribute(s) to the spectra. At this magnetic field value, the light *minus* dark spectrum was featureless, indicating that upon illumination at 200 K the cytochrome(s) do not undergo any redox changes and thus do not contribute to the light-induced changes in the ESEEM spectra.

Figure 5 shows the time domain (panels A and B) and frequency domain (panels C and D) recorded in WT* PSII (panels A and C) and D1-His332Ser PSII (panels B and D). The black traces were recorded in the dark-adapted sample, the green traces after 200 K illumination. The magenta traces are the light *minus* dark ESEEM (panels A and B) and the Fourier transform

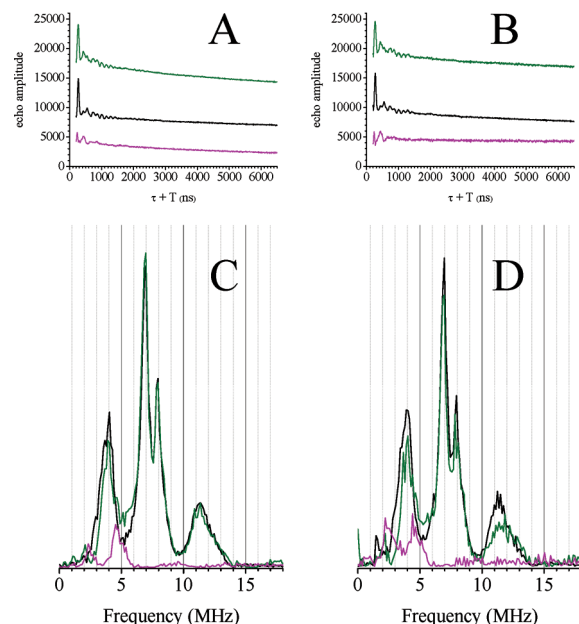


FIGURE 5: ESEEM spectra in WT* PSII and D1-His332Ser PSII. Time domain spectra (A and B) and frequency domain spectra (C and D) recorded in WT* PSII (A and C) and D1-His332Ser PSII (B and D). The black traces were recorded in the dark-adapted samples, the green traces after 200 K illumination and the magenta traces are the light *minus* dark ESEEM in panels A and B, and the Fourier transform of the light *minus* dark ESEEM in panels C and D. In panels A and B, the light *minus* dark ESEEM (magenta) were down shifted by 5000 arbitrary units for clarity. Magnetic field = 3540 gauss; temperature, 4.2 K; microwave frequency, 9.7 GHz. Shot repetition time = 8 ms.

of the light *minus* dark ESEEM (panels C and D). In panels A and B, the light *minus* dark ESEEM (magenta) were down shifted by 5000 arbitrary units for clarity.

The amplitude of the modulations arising from the multiline signal is rather weak when compared to the amplitude of the peaks at $\approx 4, 7, 8,$ and 11.5 MHz recorded in the S_1 -state and which very likely arise from the tetrapyrrole nitrogen(s) of Cyt c_{550} . These frequencies are very similar to those arising from Cyt b_{559} (59). Because of the τ value used here and the magnetic field value at which the data were recorded, the free proton frequency at $14-15$ MHz was suppressed. Fortunately, the peaks in the Fourier transform of the light *minus* dark ESEEM spectra are different from those arising from the cytochromes which confirms that the light *minus* dark spectra are free from contributions from the cytochrome signals. Figure 5C shows that small peaks at around 2.5 and 4.5 MHz are found with the WT* PSII as observed previously in *Synechocystis* 6803 (31). In the D1-His332Ser mutant (Figure 5D), the spectra are identical to those in WT* in the limit of the signal-to-noise ratio. The very small difference in the relative amplitude of the peaks at 2.5 and 4.5 MHz in the mutant and the WT* sample is not significant. Similar results were obtained at the other magnetic field values and with the other τ values (not shown).

The thermodynamic properties of the S_2 state in the mutants were investigated by measuring the thermoluminescence arising from the $S_2Q_B^-$ charge recombination. Figure 6 shows no significant change in the peak temperature of the TL glow curves arising from the $S_2Q_B^-$ charge recombination in the D1-His332Gln mutant and only a down shift by 2 °C from 50 to 48 °C in the D1-His332Ser mutant when compared to that observed for the WT* PSII. This indicates that the Em of the

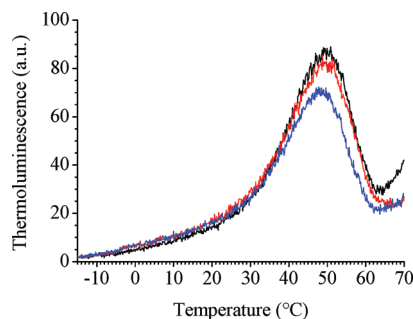


FIGURE 6: Thermoluminescence glow curves from $S_2Q_B^-$ charge recombination. TL glow curves were recorded after one flash in PSII from WT*-PSII (black trace), D1-His332Gln PSII (red trace), and D1-His332Ser PSII (blue trace).

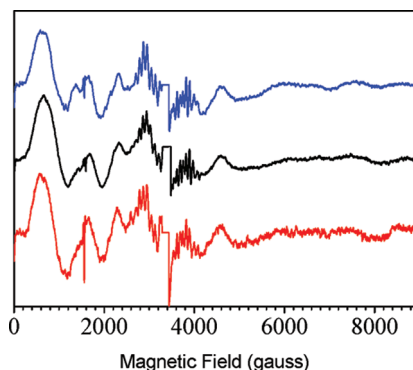


FIGURE 7: Light *minus* dark EPR spectra induced by two flashes at room temperature in the presence of 1 mM PPBQ and recorded on WT*-PSII (black spectrum), D1-His332Gln PSII (red spectrum), and D1-His332Ser PSII (blue spectrum). Sample concentration was 1.1 mg of Chl/mL. A preflash was given to synchronize all the centers in S_1 . Instrument settings: modulation amplitude, 25 G; microwave power, 20 mW; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; temperature, 8.5 K. The $Tyr_D^\bullet$ spectral region at $g \approx 2$ was deleted. The multiline signal detected after two flashes originates from centers still in S_2 and this is due to the $\approx 10\%$ misses mentioned in the text.

S_2/S_1 couple in the D1-His332 mutants is not significantly affected. Consistent with this the kinetics of $S_2Q_A^-$ charge recombination, as observed by measuring the decay of Q_A^- at 320 nm, were similar in the WT* and mutant PSII (not shown).

All the data reported above indicate that in *T. elongatus* the D1-His332(Gln, Ser) mutations do not affect significantly the thermodynamic, kinetic, or structural properties of the S_2 -state. Yet, the possibility remains about the other S_n -states and in particular the S_3 state the formation of which is associated with significant conformational changes (60–64).

To address this issue, we relied on the recently reported cw-EPR spectrum of the S_3 state in WT* PSII (40). This signal has been shown to originate from a spin $S = 3$ state. Figure 7 shows the S_3 EPR spectrum recorded in the D1-His332(Gln, Ser) mutants. The field positions of the resonances are roughly similar in the mutants and the WT*. Therefore, the zero field splitting parameters in the mutants PSII and the WT* PSII should be not significantly modified. Nevertheless, a close examination of the spectra revealed that the main feature at around 600 gauss is reproducibly down shifted by ≈ 150 gauss (Supporting Information).

The redox properties of the S_3 -state were probed by TL experiment. Figure 8 shows the TL glow curves of the $S_3Q_B^-$ charge recombination. Although the peak temperature in the D1-His332Ser PSII is only down-shifted by 2 °C (as for $S_2Q_B^-$)

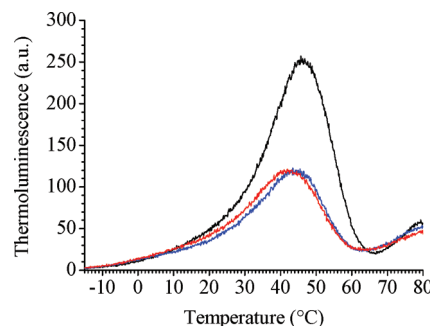


FIGURE 8: Thermoluminescence glow curves from $S_3Q_B^-$ charge recombination. TL glow curves were recorded after two flashes in PSII from WT*-PSII (black trace), D1-His332Gln PSII (red trace), and D1-His332Ser PSII (blue trace).

that in the D1-His332Gln PSII is down shifted by ≈ 4 °C from 46 to 42 °C. A qualitative estimate of the change of the Em of the S_3/S_2 couple at the origin of this 4 °C down shift can be obtained by comparing the shift in Tm observed here with those reported previously in the literature and associated with a known change in a free energy gap. For example, in *T. elongatus*, a point mutation in the vicinity of Q_A has been found to induce a shift in Tm of 9 °C and a change in free energy of ≈ 60 meV (65). Therefore, the down shift of 4 °C could correspond to an increase of ≈ 27 meV of the Em of the S_3/S_2 couple in the mutants. Numerical simulations of TL glow curves indicated that the Tm depends linearly on the ΔH of the direct route with a slope of ≈ 0.4 °C/meV (66). Such a dependency would suggest an increase in the Em of the S_3/S_2 couple in the mutants of 10 meV. The analysis of both the kinetics of the charges recombination and TL glow curves in various PSII mutants in *Synechocystis* 6803 resulted in ≈ 0.2 °C/meV (67). Therefore, by averaging the data in these three previous works (65–67) the increase of the Em of the S_3/S_2 couple in the D1-His332(Gln,Ser) mutants can be estimated to be ≈ 20 meV.

Figure 8 shows that for the $S_3Q_B^-$ charge recombination the amplitude of the TL glow curve for WT* PSII was twice that of the D1-His332(Gln, Ser) mutants. This smaller amplitude of the TL glow curve in the mutants is in agreement with an increase in the Em of the S_3/S_2 couple in these mutants (66, 67).

Alternatively, we followed the decay of the $S_3Q_B^-$ state by measuring the time-course of the S_3 EPR spectrum after two flashes (Figure 9). The samples were frozen at various times after the two flashes given at room temperature, and the amplitude of the signal at around 800 gauss was measured. Fitting the data with a single exponential decay and an offset to account for the proportion of centers in the Q_B state ($\approx 20\%$) yielded half times of ≈ 3.5 min in WT* PSII and ≈ 2.9 min in the mutants. Such a difference in the $t_{1/2}$ indicates an increase by $\approx 60 \log(3.5/2.9) = 5$ meV of the S_3/S_2 couple in the mutants. This is less than expected from the TL experiments. This difference may stem from the intrinsic complexity of the $S_3Q_B^-$ charge recombination which is a polyphasic process, the slow components of which could be more affected by the mutation than the fast phase. Indeed, one has to keep in mind that the TL experiments mainly probe the slower charge recombination processes as discussed previously (68). The number of data points in Figure 9 does not allow a reliable fit of the data with two exponentials leaving this possibility open.

It has been shown earlier that the binding properties of the two water molecules could be resolved in S_3 (47) and that these properties were strongly affected either by Ca/Sr exchange or by

some mutations in the Mn_4Ca cluster environment (16, 29, 33, 48, 49). The results above suggest that the D1-His332(Ser, Gln) mutations in *T. elongatus* slightly affect the thermodynamic and structural properties of the S_3 state, and we thus studied water exchange to examine the consequences on substrate binding.

The results from the ^{18}O exchange measurements of the WT* and D1-His332Gln mutant PSII core samples are shown for the S_3 state in Figure 10 and in Table 1. The $m/z = 34$ data reveal biphasic kinetics and the $m/z = 36$ data are monophasic and have both been shown to be characteristic for the two substrate water sites that have been seen in all other PSII samples (16, 29, 33, 47–49). The WT* material (black circles) and D1-His332Gln data (red circles) are presented in Figure 10 along with the fitted exchange kinetics (solid lines) according to eq 1 for $m/z = 34$ and eq 2 for $m/z = 36$ in Figure 10. The inset to the $m/z = 34$ data in Figure 10 shows a shorter time scale to better compare the fast phase. The exchange rates for WT* PSII were found to be $k_1 = 0.40 \pm 0.02 \text{ s}^{-1}$ and $k_2 = 18.9 \pm 1.0 \text{ s}^{-1}$ for the slow and fast phases, respectively. The D1-His332Gln mutation gave exchange rates of $k_1 = 0.15 \pm 0.01 \text{ s}^{-1}$ and $k_2 = 37 \pm 5 \text{ s}^{-1}$. The indication from these results is a measurable change in both substrate water

binding sites. The D1-His332Gln mutation results in a ~ 3 fold decrease of the slow (tightly bound) exchange rate and a ~ 2 fold increase of the fast exchange rate. Thus, both sites appear to be perturbed by the D1-His332Gln mutation. The perturbation of both the slow and fast exchange substrate sites is one indication of a direct change in the substrate binding affinity resulting from the D1-His332Gln mutation.

DISCUSSION

A number of amino acid residues have been implicated in the coordination of the Mn_4Ca cluster where the oxidation of water is catalyzed and the O–O bond is templated. Thus far, seven amino acids have been identified as possible ligands in the 3D structure (1–3). This includes residue D1-His332 which is interesting as this is likely to be the only histidine coordination to the Mn_4Ca complex. The histidine residue has interesting chemical properties associated with its pK and the interaction with the OEC is an important question to resolve. The relationships of the D1-His332 amino acid with kinetics and thermodynamic properties of the Mn_4Ca cluster were investigated in PSII from *T. elongatus* by examining the D1-His332(Gln,Ser) mutants.

In analyzing these mutants, we found both mutants grew photoautotrophically and active PSII can be purified. Oxygen evolution activity in the D1-His332(Gln, Ser) mutants was found to be approximately 80% of that measured in WT* PSII. Yet, the same mutation constructed in *Synechocystis* PCC 6803 did not allow the authors (15, 35) to purify active PSII centers. This difference suggests that the thermostability of *T. elongatus* offers an improved capacity for viability with mutation at this site. This may be the result of stronger ligation of the remaining metal ligands or it may offer an aspect of coordination compensation within the oxygen evolving complex.

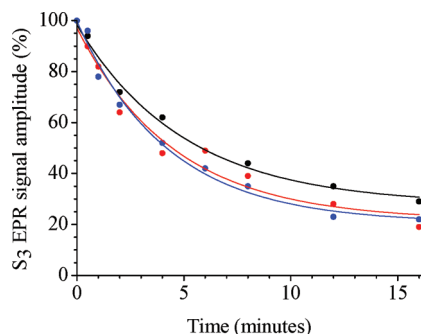


FIGURE 9: Decay at room temperature of the S_3 -state in WT* PSII (black), D1-His332Gln PSII (red), and D1-His332Ser PSII (blue). The S_3 -state was followed by recording the S_3 EPR spectrum shown in Figure 7 as a function of the time between the two flashes spaced 1 s apart and the freezing of the sample. A preflash protocol was used, but no PPBQ was added to keep Q_B^- present at time $t = 0$. Instrument settings as in Figure 7.

Table 1: Rate Constants for ^{18}O Exchange in the S_3 State of PSII Core Complexes from WT* and a Mutant of *T. elongatus*

sample	$k_1 \text{ (s}^{-1}\text{)}$	$k_2 \text{ (s}^{-1}\text{)}$
WT*	0.40 ± 0.02	18.9 ± 1.0
D1-His332Gln	0.15 ± 0.01	37 ± 5

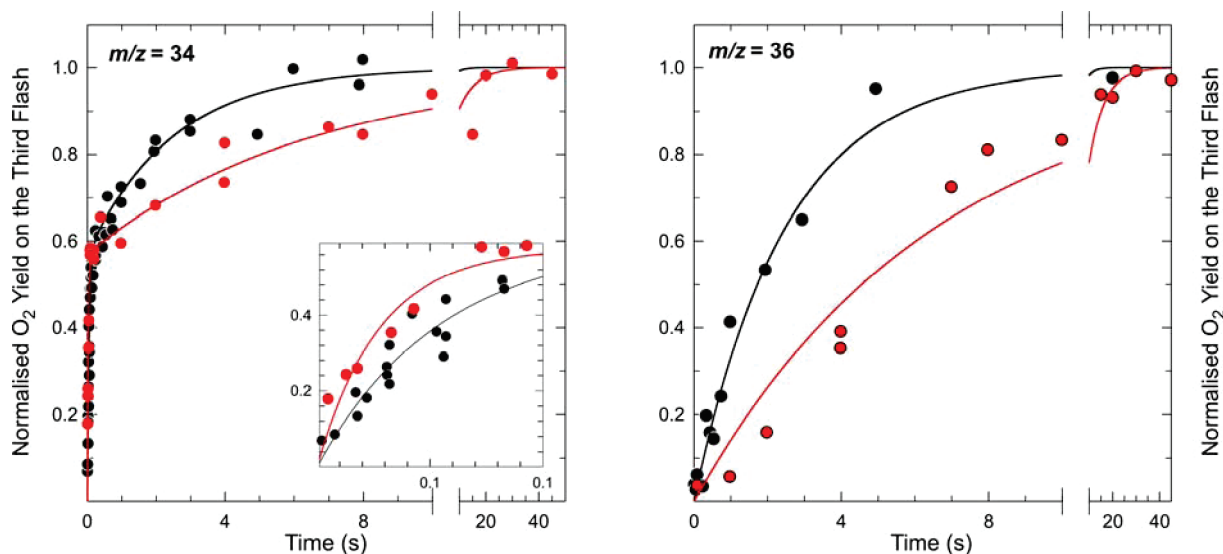


FIGURE 10: Water ^{18}O exchange measurements in the S_3 state of purified PSII from WT* and D1-His332Gln mutant. The WT* data are shown in black and the D1-His332Gln data are shown in red. The inset is a shorter time expanded to show the fast phase.

The analysis of the flash patterns (Figure 1) provides for a capacity to analyze functional centers and turnover efficiency. The flash dependence of the profile showed that the turnover efficiencies were not at all perturbed by the mutations. The absence of any major consequences of the two mutations on the turnover of the S_n -state cycle (Figure 1) under flashing conditions and the lack of significant changes in the kinetics of the $S_i \rightarrow S_{i+1}$ transition, and particularly in the kinetics of the S_3 to S_0 transition, suggest that the slight decrease in the O_2 evolution activity under continuous illumination likely results from the integration of several limited kinetics effects which escaped detection when studying separately the individual steps in the catalytic cycle. The slightly smaller S_1/S_0 ratio in the mutants upon dark adaptation could also reflect the small differences in electron transfer reactions and equilibria between the redox cofactors. In addition, we cannot totally exclude long-range effects of the mutations on the acceptor side.

On the basis of experiments reported here, it appears that the D1-His332(Gln, Ser) mutations have no effect in the S_2 state, at least for the parameters assessed in this work. This differs from the situation in *Synechocystis* 6803 in which the absence of His332 resulted in modified EPR and TL characteristics (30, 69).

A pulse-EPR study performed in the S_2 state and aiming at determining whether the D1-His332 was a ligand of the Mn_4Ca cluster (and performed before the publication of the 3D structures) (31) concluded that a histidyl nitrogen modulation observed near ≈ 5 MHz was substantially diminished in D1-His332Glu PSII. The peak at ≈ 5 MHz was shown to originate from the imidazole side chain of a histidine (70). Therefore, as proposed by the authors in ref 31, since the multiline signal was altered in the D1-His332Glu mutant, the 4.5 MHz peak could originate from the nitrogen-nucleus of another histidine, possibly D1-His337 from the structures published after the work in ref 31. In this model, the magnetic coupling between the nuclear spin of this nitrogen and the electronic spin of the Mn_4Ca cluster would be modified by the same structural change as that at the origin of the S_2 multiline modification in the D1-His332Glu mutant. In *T. elongatus* in which the D1-His332Ser mutant does not exhibit a S_2 multiline modification it is shown here that the frequency peaks at around 2.5 and 4.5 MHz do not decrease. This means that (i) the peaks at 2.5 and 4.5 MHz observed in pulse-X-band EPR do not originate from the nitrogen(s) of the imidazole ring of D1-His332, (ii) the histidyl nitrogen(s) of D1-His332 were not detected by ESEEM performed at X-band, (iii) the mutation does not change the magnetic coupling between the multiline signal and this/these nitrogen(s), (iv) the 2.5 and 4.5 MHz could originate from the nitrogen(s) of the imidazole ring of D1-His337, (v) the results in *Synechocystis* 6803 likely originate from the structural changes at the origin of the S_2 multiline signal modifications which would be a kind of secondary effect which would remain to be explained.

With the exception of D1-His332 (see above) none of the six other potential ligands identified by 3D crystallography, that is, D1-Ala344 (24), D1-Glu189 (25, 26), D1-Asp342 (27), D1-Asp170 (28), D1-Glu333 (35), and CP43-Glu354 (29), seem to be essential for the activity in *Synechocystis* 6803. One may speculate that they have other roles in the assembly, stability, and/or the repair of PSII rather than a direct role for oxygen evolution mechanism itself (see introduction). However, it is also possible that coordination is viable with these ligands and the mutations are rescued. From the present work, D1-H332 appears also not essential for the water oxidation mechanism.

In *Synechocystis* 6803, for the three mutants D1-Asp342Asn (27), D1-Asp170His (28), and D1-Glu189(Gln, Arg) (25), see however ref 26, in which the FTIR spectra have been recorded in all the S -state transitions, none of these mutations induces significant spectral change. This was interpreted by a model in which the Mn ions ligated by these amino acids were not oxidized during the S_n -state cycle. It was, for example, proposed that the Mn ion oxidized in the S_1 to S_2 transition was that ligated by D1-Ala344 (71). Nevertheless, it is striking that such a sensitive method is blind for most of the mutants around the Mn_4Ca cluster.

Similarly, the S_2 EPR multiline signal has been found to be not susceptible to the change of six of its ligands. This could suggest that this is the Mn_4Ca cluster, provided it is correctly assembled, which structures the protein around it. It seems that the missing ligands in the 3D structure, possibly water molecules, are more important in maintaining the Mn_4Ca structure, at least in the S_2 state, than thought until now. It is indeed difficult to explain why the Ca^{2+}/Sr^{2+} exchange affects so strongly the S_2 multiline signal (46) whereas the substitution of a glutamine for D1-Glu189 which, from the 3D structures (1–3) is a bidentate ligand between the Ca^{2+} and one Mn ion, has no effect.

In *Synechocystis* 6803, the D1-His332 mutants were also studied by thermoluminescence experiments. These measurements were done in whole cells (69). As in purified PSII, the centers were found to be inhibited after $S_2Tyr_Z^*$ formation in the large majority of PSII centers, and the results were interpreted by a down shift of the Em of the S_2/S_1 couple in the D1-His332(Asp, Glu, Ser) mutants by 75, 50, and 7 mV, respectively (69). In *T. elongatus*, in agreement with the lack of EPR changes in the S_2 state, no change in the TL properties of the D1-His332 mutants was observed.

In *T. elongatus*, in contrast to S_2 , differences have been detected here in the S_3 state in the D1-His332(Gln, Ser) mutants. The S_3 EPR signal is slightly modified and the E_m of the S_3/S_2 couple is increased by ≤ 20 mV.

The similar field positions of the resonances in the S_3 signals in WT* PSII and the D1-His332 mutants strongly suggest that the zero field parameters are not significantly affected by the mutations. Nevertheless, reproducible differences were observed. Several possibilities can be put forward to explain such small differences; (i) the contribution of another signal which would suggest a possible heterogeneity in the S_3 state of the mutants. This would not be surprising since heterogeneities have been already observed in the S_2 multiline signal (72); (ii) small changes in Mn hyperfine interactions from the Mn nuclei which seem partially resolved in some spectra although with a low signal-to-noise ratio (see Supporting Information). So far we could not find conditions in which the S_3 EPR signal was detectable by 9 GHz pulse EPR. This likely originates from too fast relaxation times due to the high spin value, $S = 3$. This also precludes the detection of possible couplings between the nitrogens of D1-His332 and the Mn_4Ca cluster. Further work will be required to solve all these questions.

Water binding is perturbed with both substrate binding sites in the S_3 state in the D1-His332Gln mutant. The tightly bound water at the slow exchange site is retained more tightly in the D1-His332Gln mutation with a ~ 3 -fold slowing in exchange rate (Table 1). This retention could be expected to correlate with an increase in the formal charge of the metal binding site (16, 73). The nature of the tightly bound substrate water is consistent with a metal site exhibiting unimolecular exchange based on a

correlation between the exchange rate and activation energy (47). Indeed, the tightly bound substrate water is also retained more weakly in Sr^{2+} substituted PSII centers (74, Hillier and Boussac unpublished). The change in the rate in D1-His332Gln for the tightly bound substrate water molecule can be explained as an increased in charge associated with the Mn_4Ca complex. The obvious explanation from this is a change in the ligand coordination sphere.

Alternatively, the D1-His332 could be a supporting ligand to a substrate water binding metal. However, it is unlikely that mutation of D1-His332 if serving as a ligand to the Mn_4Ca complex goes uncompensated. That is to say that the mutation will not likely result in an open empty coordination site. Either a water molecule will instead bind, or the $-\text{C}=\text{O}$ or $-\text{NH}_2$ donors that are present in the Gln side chain will act as donors.

The exchange rate of the faster exchangeable water increased in the D1-His332Gln mutant which is indicative of a weaker substrate binding site. The effect of the mutation on the two water exchange rates differs therefore in amplitude and in direction which indicates a dissimilar perturbation. We know less about the origin of the fast exchanging substrate water, but recent unpublished results do suggest an association with a metal site coordinated by CP43-Glu354 (Hillier, unpublished).

Ultimately, it is attractive to postulate that the D1-His332 is a direct ligand to a water substrate binding metal in S_3 , but there remains the possibility for perturbations in the second coordination sphere that may manifest in changes regarding substrate exchange.

All the results above suggest that the D1-His332 is more involved in S_3 than in S_2 . This could be one element of the conformational changes put forward in the S_2 to S_3 transition from the S-state dependence of Ca-depletion (63), EXAFS spectroscopy (64), differences of activation energy in S_2 and S_3 (60, 61), and different reactivity's to exogenous reductants (62).

ACKNOWLEDGMENT

Yu Matsuda and Thanh-Lan Lai are acknowledged for technical help. We would like to thank Takumi Noguchi and Hiroyuki Suzuki for setting up Thermoluminescence machine and helpful discussions and also Bill Rutherford for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Fitting of the flash induced absorption changes at 292 nm and EPR spectra with an expended magnetic field scale. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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