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Probing the role of Arginine 323 of the D1 protein in Photosystem II function[‡]

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[‡] This paper is dedicated to the memory of our colleague Jean-Marc Ducruet who sadly passed away in summer 2019.

The Mn₄CaO₅ cluster of Photosystem II (PSII) advances sequentially through five oxidation states (S₀ to S₄). Under the enzyme cycle, two water molecules are oxidized, O₂ is generated and four protons are released into the lumen. Umena et al (2011) have proposed that, with other charged amino acids, the R323 residue of the D1 protein could contribute to regulate a proton egress pathway from the Mn₄CaO₅ cluster and Tyr_Z via a proton channel identified from the 3D structure. To test this suggestion, a PsbA3/R323E site-directed mutant has been constructed and the properties of its PSII have been compared to those of the PsbA3-PSII by using EPR spectroscopy, polarography, thermoluminescence and time-resolved UV-visible absorption spectroscopy. Neither the oscillations with a period four nor the kinetics and S-state-dependent stoichiometry of the proton release were affected. However, several differences have been found: *i*) the P₆₈₀⁺ decay in the hundreds of ns time domain was much slower in the mutant, *ii*) the S₂Q_A⁻/DCMU and S₃Q_A⁻/DCMU radiative charge recombination occurred at higher temperatures and *iii*) the S₀Tyr_Z[•], S₁Tyr_Z[•], S₂Tyr_Z[•] split EPR signals induced at 4.2 K by visible light from the S₀Tyr_Z, S₁Tyr_Z, S₂Tyr_Z, respectively, and the

($S_2\text{Tyr}_Z^*$) induced by NIR illumination at 4.2 K of the $S_3\text{Tyr}_Z$ state differed. It is proposed that the R323 residue of the D1 protein interacts with Tyr_Z likely via the H-bond network previously proposed to be a proton channel. Therefore, rather than participating in the egress of protons to the lumen, this channel could be involved in the relaxations of the H-bonds around Tyr_Z by interacting with the bulk, thus tuning the driving force required for Tyr_Z oxidation.

Abbreviations — CHES, *N*-Cyclohexyl-2-aminoethanesulfonic acid; Chl, chlorophyll; $\text{Chl}_{D1}/\text{Chl}_{D2}$, accessory Chl's on the D1 or D2 side, respectively; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LS, low spin; PSII, Photosystem II; PPBQ, phenyl *p*-benzoquinone; HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; HS, high spin; MES, 2-(*N*-morpholino) ethanesulfonic acid; NIR, near infrared; P_{680} , primary electron donor; P_{D1} and P_{D2} ; Chl monomer of P_{680} on the D1 or D2 side, respectively, Phe_{D1} and Phe_{D2} , pheophytin on the D1 or D2 side, respectively; Q_A , primary quinone acceptor; Q_B , secondary quinone acceptor; S_2^{LS} , low spin S_2 state; S_2^{HS} , high spin S_2 state; Tyr_D , redox active tyrosine 160 of D2; Tyr_Z , redox active tyrosine 161 of D1; TL, thermoluminescence; WT*3, *T. elongatus* mutant strain containing only the *psbA*₃ gene and a His₆-tag on the C-terminus of CP43.

Introduction

The light-driven water-splitting carried out by Photosystem II (PSII) in cyanobacteria, algae, and plants is the major input of energy into the living world. Indeed, it is responsible for the production of the atmospheric O₂ that is essential for aerobic organisms and it is the first step in the production of food, fibers and fossil fuels. In cyanobacteria, the PSII complex consists of 17 trans-membrane and 3 extrinsic membrane proteins (Umena et al. 2011, Suga et al. 2015). X-ray crystal structure revealed cofactors and metal ions: 35 chlorophylls *a* (Chl*a*), 2 pheophytins (Phe), 2 hemes, 1 non-heme iron, 2 plastoquinones (Q_A and Q_B), the Mn₄CaO₅ cluster, 2 Cl⁻, 12 carotenoids and 25 lipids. Most of cofactors involved in the water oxidation and electron transfer bind to the reaction center subunits, D1 (also called PsbA) and D2 (also called PsbD).

The electron transfer reactions start with the excitation of one of the chlorophylls of the antenna upon the absorption of a photon. The excitation is then transferred to the

photochemical trap which consists of four chlorophylls, P_{D1}, P_{D2}, Chl_{D1}, Chl_{D2}. A charge separation then occurs resulting ultimately in the formation of the Chl_{D1}⁺Phe_{D1}⁻ and then in the P_{D1}⁺Phe_{D1}⁻ radical pair states see for example (Holzwarth et al. 2006, Romero et al. 2010, Müh et al. 2017, Duan et al. 2017). Then, P_{D1}⁺ oxidizes Tyr_Z, the Tyr161 of the D1 polypeptide, which in turn oxidizes the Mn₄CaO₅ cluster, for example (Shen 2015) for a review. The electron on Phe_{D1}⁻ is then transferred to Q_A, the primary quinone electron acceptor and then to Q_B, the second quinone electron acceptor. Whereas Q_A can be only singly reduced under normal conditions, Q_B accepts two electrons and two protons before leaving its binding site (Bouges-Bocquet 1975, see also Fufezan et al. 2005, Boussac et al. 2010, Sedoud et al. 2011).

The Mn₄CaO₅ cluster is sequentially oxidized following each charge separation, thus cycling through five redox states denoted S_{*n*}, where *n* stands for the number of stored oxidizing equivalents (Joliot and Kok 1975, Kok et al. 1970). Since the state stable in the dark is S₁, the S₄-state is formed after the 3rd flash of light given on dark-adapted PSII. In the S₄-state, the two water molecules bound to the cluster are oxidized, the O=O bond is formed, O₂ is released and the S₀-state is regenerated. During the enzyme cycle, the increase in charge resulting from electron abstraction from the Mn₄CaO₅ cluster is compensated for by proton releases which help to maintain its redox potential below that of Tyr_Z[•]/Tyr_Z (Rappaport and Lavergne 2001, Klauss et al. 2012, Siegbahn and Lundberg 2005). At pH 6.5, the stoichiometry for the proton release into the bulk is close to 1, 0, 1, 2 in the S₀ → S₁, S₁ → S₂, S₂ → S₃ and S₃ → S₀ transitions, respectively (Saphon and Crofts 1977, Fowler 1977, Rappaport and Lavergne 1991, Suzuki et al. 2009, Schlodder and Witt 1999, Förster and Junge 1985).

The proton movements occurring during the S_{*n*}-state cycle are strongly influenced both by the hydrogen (H)-bonding network involving the first coordination sphere of the Mn₄CaO₅ cluster, and also by the H-bonding networks that extend well outside the first coordination sphere (Debus 2015, Noguchi 2016, Bao et al. 2013, Saito et al. 2015, Siegbahn 2014, Kim et al. 2018). For identifying which amino acids outside the first coordination sphere of the Mn₄CaO₅ are involved in the networks tuning the properties of the cluster, a great number of site-directed mutants have been constructed and studied. Among the residues studied and belonging to either D1, or D2 or CP43 or CP47 there are: D1/E65, D2/E312 and D1/E329 (Service et al. 2010), D1/R334, D1/Q165, D2/D307, D2/E308, D2/D310, D2/D323 (Service et al. 2014) and D2/K317 (Pokhrel et al. 2013, Suzuki et al. 2013), D1/N181 (Pokhrel et al. 2015), D1/N298 (Nagao et al. 2017), D1/N87 (Banerjee et al. 2018), D1/S169

(Ghosh et al. 2019a), D1/P173 (Sugiura et al. 2014b), CP43/R357 (Ghosh et al. 2019b) and D1/V185 (Bao and Burnap 2015, Sugiura et al. 2018).

Based on the 3D crystal structures, several channels have been proposed for the egress of the protons from the Mn_4CaO_5 complex into the bulk. For example, the D1/N298, D1/N322, D1/D319, D1/H304, PsbV/K129 residues and some water molecules have been proposed to constitute a channel from the D1/H190, H-bonded to Tyr_Z, to the lumen (Umena et al. 2011; Fig. 1A). Carboxyl terminus of PsbV/Y137 is sandwiched between this channel and the D1/R323 residue (Fig. 1B). Surprisingly, the D1/R323E mutation has been shown to dramatically impair the PSII function in *Chlamydomonas reinhardtii* (Makarova et al. 2007). However, the inhibited reaction at the origin of this loss of activity in the mutant has not been precisely determined.

In the present work, the D1/R323E mutant has been constructed in *Thermosynechococcus elongatus* and has been characterized by a combination of techniques like O₂ polarography by using a Clark-type electrode under continuous illumination, EPR spectroscopy at helium temperatures, thermoluminescence, UV-visible time-resolved absorption changes spectroscopy including proton release detection with bromocresol purple as a dye under flash illumination.

Materials and Methods

Construction of the PsbA3/R323E mutant and Photosystem II preparation

The *Thermosynechococcus elongatus* strain used was the $\Delta psbA_1$, $\Delta psbA_2$ deletion mutant, called WT*3 (Sugiura et al. 2008), constructed from the *T. elongatus* 43-H strain that had a His₆-tag on the carboxy terminus of CP43 (Sugiura and Inoue 1999). For the construction of the D1/R323E site-directed mutant, the positions at +967, +968 and +969 in *psbA₃* were substituted on a plasmid DNA in order to introduce a R323E mutation and to create a restriction site for *AseI* by a Quick-Change Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) as shown in Fig. 2A. After introduction of the plasmid DNA into the host cells by using an electroporator (BioRad gene pulser), mutant cells were selected from colonies on DTN agar plate containing 5 $\mu\text{g mL}^{-1}$ of chloramphenicol, 25 $\mu\text{g mL}^{-1}$ of spectinomycin, 10 $\mu\text{g mL}^{-1}$ of streptomycin, and 40 $\mu\text{g mL}^{-1}$ of kanamycin as previously described (Sugiura et al. 2008). Fig. 2C shows the results of the genomic DNA analysis performed around the site-directed mutation in segregated cells. After PCR amplification of the mutated region by using a forward primer (Fw; 5'–

ACGGTTACAAATTTGGTCAAGAGGA–3') and a reverse primer (Rv; 5'–GAGCTCCGGAAACTAGCCGTTGATGCTGGG–3') (Fig. 2B), the PCR products were digested with *AseI*. As shown in Fig. 2C, amplified 391-bp DNA fragments (lane 4) were completely digested by *AseI* in the R323E mutant genome (lane 5). In contrast to the R323E genome, the amplified 391-bp DNA fragments in the WT*3 genome could not be digested with *AseI* (lanes 2 and 3). In addition, the genomic DNA sequence of the open reading frame of *psbA₃* was confirmed by nucleotides sequencing by using a GenomeLab GeXP DNA sequencer (Beckman Coulter).

PSII purification was achieved as previously described (Sugiura et al. 2014). The PsbA3/E130Q and PsbA3/P173M mutants used in the thermoluminescence experiment have been previously described in (Sugiura et al. 2014a, 2014b).

In some PSII preparations, it has been observed with SDS-PAGE that the Cyt c 550 content in purified PsbA3/R323E-PSII could be affected (not shown). In these cases, the PsbA3/R323E-PSII PSII lacking some Cyt c 550 was not used. This observation, however, likely means that the R323E mutation may affect the binding affinity of the Cyt c 550 to PSII. We have also controlled the Cyt c 550 signal in the purified PsbA3/R323E-PSII by EPR. Indeed, when this cytochrome is slightly destabilized from its binding site but still bound to PSII, a slight shift of the EPR resonances can be detected. In the purified PsbA3/R323E-PSII, which was used in the present study, the Cyt c 550 EPR signal was identical to that in PsbA3-PSII (not shown) thus showing that the cytochrome was in its native binding site.

Oxygen evolution

Oxygen evolution activity of thylakoid membranes under continuous illumination was measured at 25°C by polarography using a Clark type oxygen electrode (Hansatech) with saturating white light through infrared and water filters. The initial rate was measured in the presence of 0.5 mM 2,6-dichloro-*p*-benzoquinone (DCBQ), dissolved in DMSO, as an electron acceptor. The medium used contained 15 mM CaCl₂, 15 mM MgCl₂, 20 mM NaCl and 20 mM Good's buffer which was MES for pH 5.0, 5.5, 6.0, 6.5 and 7.0, HEPES for pH 7.5 and 8.0, and CHES for pH 8.5.

UV-visible time-resolved absorption change spectroscopy

Absorption changes measurements have been performed with a lab-built spectrophotometer (Béal et al. 1999) in which the absorption changes were sampled at discrete times by short analytical flashes. These analytical flashes were provided by an optical

parametric oscillator (Horizon OPO, Amplitude Technologies) pumped by a frequency tripled Nd:YAG laser (Surelite II, Amplitude Technologies), producing monochromatic flashes (355 nm, 2 nm full-width at half-maximum) with a duration of 5 ns. Actinic flashes were provided by a second Nd:YAG laser (Surelite II, Amplitude Technologies) at 532 nm, which pumped an optical parametric oscillator (Surelite OPO plus) producing monochromatic saturating flashes at 695 nm with the same pulse-length. The two lasers were working at a frequency of 10 Hz and the time delay between the laser delivering the actinic flashes and the laser delivering the detector flashes was controlled by a digital delay/pulse generator (DG645, jitter of 1 ps, Stanford Research). The path-length of the cuvette was 2.5 mm.

For the $\Delta I/I$ measurements at 291 nm, 440 nm, 424 nm and 432 nm, the O₂ evolving PSII samples were diluted in 1 M betaine, 15 mM CaCl₂, 15 mM MgCl₂, and 40 mM MES (pH 6.5). PSII samples were then dark-adapted for ~ 1 h at room temperature (20–22°C) before the addition of 0.1 mM phenyl *p*-benzoquinone (PPBQ) dissolved in dimethyl sulfoxide. The chlorophyll concentration of all the samples was ~ 25 μg of Chl mL⁻¹.

For proton release/uptake detection, a solution containing 1 M betaine, 15 mM CaCl₂, 15 mM MgCl₂ and 150 μM bromocresol purple as indicator dye was used. The pH was adjusted to 6.3, the pK_a of the dye, with NaOH. The solution containing the dye was prepared several days before the measurements and the pH adjusted again before the experiments. Such a procedure minimizes the drift in the base line at 575 nm during the experiment. After dark adaptation of the PSII in this medium for 1 h at room temperature, 100 μM PPBQ (in DMSO) and 100 μM potassium ferricyanide were added.

After the $\Delta I/I$ measurements, the absorption of each diluted batch of PSII samples was precisely controlled to avoid errors due to the dilution of concentrated samples and the $\Delta I/I$ values were normalized to $A_{673} = 1.75$, with $\epsilon \sim 70 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 674 nm for dimeric PSII (Müh and Zouni 2005).

EPR spectroscopy

X-band cw-EPR spectra were recorded with a Bruker Elexsys 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 temperature was provided by a neodymium:yttrium–aluminum garnet (Nd:YAG) laser (532 nm, 550 mJ, 8 ns Spectra Physics GCR-230-10). Illumination at 198 K with visible light was done in a non-silvered Dewar filled with ethanol cooled with dry ice for approximately 5-10 seconds with a 800 W tungsten lamp filtered by

water and infrared cut-off filters. Illumination at 4.2 K was done in the EPR cavity using a low-voltage halogen lamp (24V, 250W, Philips Type 13163) filtered by water and infrared cut-off filters. Near infrared (NIR) illumination was provided by a laser diode emitting at 813 nm (Coherent, diode S-81-1000C) with a power of 600-700 mW at the level of the sample. The samples were synchronized in the S_1 -state with one pre-flash (Styring and Rutherford 1987). Then, after a further 1 h dark-adaptation at room temperature the samples were frozen in the dark to 198 K in a dry ice ethanol bath and then transferred to 77 K in liquid N_2 . Prior to recording the spectra the samples were degassed at 198 K.

Thermoluminescence measurements

Thermoluminescence (TL) glow curves were measured with a lab-built apparatus (Ducruet 2003, Ducruet and Vass 2009). PSII samples were diluted in 1 M betaine, 40 mM MES, 15 mM $MgCl_2$, 15 mM $CaCl_2$, pH 6.5 and then dark-adapted for at least 1 h at room temperature before the addition of DCMU (100 μ M) dissolved in ethanol. Flash illumination was done at $-10^\circ C$ by using a saturating xenon flash as indicated in the text. The constant heating rate was $0.4^\circ C/s$ in all cases. It has been checked after the dilutions that the PSII samples had the same OD at 673 nm equal to 0.70 i.e. $\sim 10 \mu g \text{ Chl mL}^{-1}$.

Results

Fig. 3 shows the pH dependence of O_2 evolution measured under continuous illumination, in the presence of DCBQ, in PsbA3/R323E-thylakoids (red trace) and in PsbA3-thylakoids (black trace). In PsbA3-thylakoids the activity was maximum at pH 7.0 as previously observed in wild type-purified PSII in the presence of DCBQ, the electron acceptor used here (Boussac et al. 2004). In PsbA3/R323E-thylakoids, the optimum pH value was slightly down-shifted by 0.5 pH unit to pH 6.5. The maximum activities in PsbA3-thylakoids at pH 7.0 and PsbA3/R323E at pH 6.5 were very similar with $\sim 330\text{-}310 \mu\text{mol } O_2 (\text{mg Chl})^{-1} \text{ h}^{-1}$. In WT*3 sample, the pH value of 7.0 has been shown to be the pH at which the activity is the highest in the presence of DCBQ whereas it is at pH 6.5 in the presence of PPBQ (discussed in Sugiura et al. 2018).

Fig. 4 shows the amplitude of the absorption changes upon flash illumination in 3 different experiments recorded in the PsbA3-PSII (black symbols) and in the PsbA3/R323E-PSII mutant (red symbols). In Panel A, the $\Delta I/I$ measurements were done at 291 nm and 100 ms after each flash of the sequence, i.e. after the reduction of Tyr_{rZ}^\bullet by the Mn_4CaO_5 cluster was complete (Lavergne 1991, Rappaport et al. 1994). This wavelength corresponds to an

isosbestic point for Q_A^-/Q_A and $PPBQ^-/PPBQ$, the added electron acceptor, and is in a spectral region where the absorption of the Mn_4CaO_5 cluster depends on the S_n -states (Lavergne 1991). A very similar oscillating pattern with a period of four was clearly observed for both samples which indicates that the activity under flash illumination in the mutant is not altered.

Panel B in Fig. 4 shows the absorbance changes of the dye bromocresol purple (Nilsson et al. 2014, Sugiura et al. 2018) under flash illumination. The $\Delta I/I$ were measured 10 ms after each flash of the sequence given to PsbA3-PSII (black symbols) and to PsbA3/R323E-PSII (red symbols), i.e. before the slow “uptake” from unknown origin occurs (discussed in Nilsson et al. 2014, Sugiura et al. 2018). After the first flash, the increase in the $\Delta I/I$ mainly corresponds to a proton uptake which occurs consecutively to the reduction of the non-heme iron by Q_A^- . After the second flash, the negative $\Delta I/I$ corresponds to the release of one proton in the center which progress from S_2 to S_3 . After the third flash, the $\Delta I/I$ amplitude was approximately twice that after the second flash in agreement with the release of two protons in the S_3 to S_0 transition. Due to the misses, the proton release after the fourth flash, which triggers the S_0 to S_1 transition, was slightly smaller than after the second flash. After the fifth flash, which triggers the S_1 to S_2 transition in a second enzyme cycle, the proton release was not far from zero as expected. Importantly, the oscillating pattern in the PsbA3/R323E-PSII mutant was similar to that in the PsbA3-PSII sample thus indicating a similar stoichiometry for the proton release in the S-state cycle in the two samples.

Panel C in Fig. 4 shows the $\Delta I/I$ amplitude of the difference, 440 nm-*minus*-424 nm, after each flash of a sequence given to dark-adapted PsbA3-PSII (black symbols) and to PsbA3/R323E-PSII (red symbols). This experiment probes the electrochromic band-shifts in the Soret region of the P_{D1} absorption spectrum undergone upon the oxidation of the Mn_4CaO_5 cluster (discussed in detail in Rappaport et al. 1994, Boussac et al. 2020). Moreover, it has been previously documented that the amplitude of the 440 nm-*minus*-424 nm difference, in a flash sequence, oscillated with a period of four with no contribution due to Q_A^- formation (Rappaport et al. 1994). Indeed, at these two wavelengths, the electrochromism due to Q_A^- equally contributes. The oscillations with a period of four are clearly detectable in Fig. 4C with the same pattern in both samples with the largest negative absorption change on the 3rd flash and the largest positive absorption change, excluding the first flash, on the 5th flash.

In conclusion, from the experiments reported in Fig. 4, the oscillating pattern of the Mn_4CaO_5 cluster in the PsbA3/R323E mutant is very similar, if not identical, to that in the PsbA3-PSII.

Fig. 5 shows the thermoluminescence (TL) emitted after one flash given at -10°C in the presence of DCMU followed by an incubation for 30 s at -10°C before the start of the heating ramp and the recording of the TL signal. The black trace corresponds to PsbA3-PSII, the red trace to PsbA3/R323E-PSII, the blue trace to PsbA3/E130Q-PSII (Sugiura et al. 2014a) and the green trace to PsbA3/P173M-PSII (Sugiura et al. 2014b). The PsbA3/E130Q-PSII and PsbA3/P173M-PSII have been used for reasons thereafter explained. It has been checked in a separate experiment (not shown) that the amplitude of the TL signal recorded upon a dark period of 30 s, at -10°C , following the flash was similar to that upon a delay of only 1 s. This indicates that the $S_nQ_A^-/\text{DCMU}$ state did not decay at -10°C during the 30 s separating the flash from the start of the heating ramp. This delay was necessary to allow us to add the DCMU *after* the flash illumination in the experiments described thereafter.

The peak temperature of the TL curve corresponding to the $S_2Q_A^-/\text{DCMU}$ charge recombination was significantly up-shifted by $\sim 10^{\circ}\text{C}$, from $\sim 14^{\circ}\text{C}$ in PsbA3-PSII to $\sim 24^{\circ}\text{C}$ in PsbA3/R323E-PSII. This up-shift could indicate a higher stability, i.e. a lower energy level of the Mn_4CaO_5 cluster in S_2 state. Alternatively, it could originate from an increase in the E_m of the $\text{Tyr}_Z^{\bullet}/\text{Tyr}_Z$ couple. Indeed, an increase in the energy level of Tyr_Z would make the oxidation of Tyr_Z by S_2 , which is the first step in the recombination process on the donor side, more difficult thus resulting in an increase of the peak temperature. This increase would also explain the slightly larger TL amplitude since a more oxidant Tyr_Z^{\bullet} will favour the repopulation of P_{680}^+ which constitutes the $P_{680}^+\text{Phe}_{D1}^-$ radical pair precursor of the luminescent $^*\text{Chl}_{D1}$ species (Rappaport and Lavergne 2009, Takegawa et al. 2019). In the PsbA3/P173M-PSII mutant that has a modified Tyr_Z environment (Sugiura et al. 2014b), the TL curve corresponding to the $S_2Q_A^-/\text{DCMU}$ charge recombination was also significantly upshifted by $\sim +16^{\circ}\text{C}$ with an increase in the TL emission. The small peak at $\sim 67^{\circ}\text{C}$ observed in all the samples very likely originate from centers in which a $\text{Tyr}_D^{\bullet}Q_A^-/\text{DCMU}$ recombination occurs (Johnson et al. 1994).

Panels A and B in Fig. 6 show the TL curves recorded after one flash and two flashes, respectively, given at -10°C in the absence of DCMU followed by an incubation for 30 s at -10°C before the start of the heating ramp. For the $S_2Q_B^-$ charge recombination (Panel A) the differences in the peak temperature between the 4 samples are much less pronounced than for the $S_2Q_A^-/\text{DCMU}$ charge recombination. This shows that the large energy gap between the Q_A^-/Q_A and the Q_B^-/Q_B couples shade off, in part, the effects of the mutation on the electron donor side. This is well illustrated by the TL in the PsbA3/E130Q-PSII mutant in which the

much greater amplitude of the TL signal and the higher peak temperature for the $S_2Q_A^-$ /DCMU charge recombination originates from a decrease of the energy gap by ~ 30 meV (Sugiura et al. 2014a) between the precursor radical pairs, $(Chl_{D1}P_{D1})^+Phe_{D1}^-$, and the luminescent state $*Chl_{D1}$. Therefore, for the recording of the TL after two flashes, i.e. in the S_3 -state, but in the presence of Q_A^- instead of Q_B^- , a different protocol has been used in which the DCMU has been added *after* two flashes. For a comparison with the data in Fig. 5, an experiment was also performed by adding the DCMU *after* one flash. The delay of 30 s between either the one-flash illumination or the two-flash illumination and the start of the heating ramp has been adjusted to have enough time for adding the DCMU. This delay also allows the equilibrium $Q_AQ_B^- + DCMU \leftrightarrow Q_A^-/DCMU$ to be reached, something that has been shown to be slow in plant PSII at pH 6.5 (Lavergne 1982).

Panels C and D in Fig. 6 shows the TL curves after one and two flashes, respectively, followed by the addition of DCMU. After one flash, the differences seen in Fig. 5 when DCMU was added before the flash were kept. However, all the temperatures corresponding to the peaks of the TL were slightly up-shifted when compared to the situation in which DCMU was present before the illumination. This suggests that a relaxation occurred either in the Q_A^- state when Q_B is present or in the Q_B^- state and that this relaxation process does not revert upon the formation of the $Q_A^-/DCMU$ state from the $Q_AQ_B^-$ state. In these experiments, the amplitudes of the curves were not largely modified because about half of the centers were in the Q_B^- state before the flash and the addition of DCMU, for example (Boussac et al. 2010). Therefore, the proportion of closed centers did not significantly varied depending on the flash number (either 1 or 2) and the timing for the addition of DCMU. When DCMU was added after two flashes, the TL curve resulting from the $S_3Q_A^-/DCMU$ charge recombination was up-shifted in the PsaA3/R323E mutant by $\sim 4^\circ\text{C}$ from ~ 17 to $\sim 21^\circ\text{C}$.

From the data in Figs 3 and 4, the proposed changes in the redox properties of Tyr_Z induced by the R323E mutation did not affect, to a detectable level, the efficiency of the S-state transitions. To determine if these redox changes have nevertheless other kinetic consequences the experiments described below have been performed.

Firstly, the absorption changes at 291 nm in PsaA3/R323E-PSII were measured in the μs to ms time range after the first flash (black), the second flash (red), the third flash (blue) and fourth flash (green) in Fig. 7A. At this wavelength, only minor absorption changes are detectable after the second flash and the fourth flash because the $\Delta I/I$ changes associated with the $S_2\text{Tyr}_Z^\bullet \rightarrow S_3\text{Tyr}_Z$ and $S_0\text{Tyr}_Z^\bullet \rightarrow S_1\text{Tyr}_Z$ transitions are small. This, thus, precludes a

reliable kinetic analysis of the S_2 to S_3 and S_0 to S_1 transitions (Rappaport et al. 1994). On the other hand, because these two transitions only weakly contribute to the absorption changes at this wavelength, the half-times of the $S_1\text{Tyr}_Z^\bullet \rightarrow S_2\text{Tyr}_Z$ and $S_3\text{Tyr}_Z^\bullet \rightarrow S_0\text{Tyr}_Z$ transitions may be reliably determined from the raw data without any deconvolution procedures. After the first flash, the kinetics of the $S_1\text{Tyr}_Z^\bullet$ to $S_2\text{Tyr}_Z$ transition with a $t_{1/2}$ of $\sim 40 \mu\text{s}$ is very similar to that in PsbA3-PSII (e.g. Sugiura et al. 2018). After the third flash, the kinetics of the $S_3\text{Tyr}_Z^\bullet \rightarrow S_0\text{Tyr}_Z$ transition includes a lag phase attributed to structural rearrangements in an $S_3\text{Tyr}_Z^\bullet \rightarrow (S_3\text{Tyr}_Z^\bullet)'$ transition in which a proton release occurs before the $(S_3\text{Tyr}_Z^\bullet)' \rightarrow S_0\text{Tyr}_Z$ transition occurs (e.g. Rappaport et al. 1994, Zaharieva et al. 2016, Sugiura et al. 2018). Fig. 7A shows that neither the lag phase with a length of 100-200 μs nor the $(S_3\text{Tyr}_Z^\bullet)' \rightarrow S_0\text{Tyr}_Z$ transition with a $t_{1/2}$ of ~ 1 -2 ms were affected by the mutation.

Secondly, the time-resolved flash dependence of the proton release was investigated. Fig. 7B shows the absorption changes at 575 nm, which are associated with changes in the protonation state of bromocresol purple, after the first four flashes given to a dark-adapted PsbA3/R323E-PSII. At 575 nm, the unprotonated form of the dye has the largest extinction coefficient so that a light-induced decrease in absorbance reflects a proton release by PSII. The kinetics shown in Fig. 7B were recorded after the first flash (black), the second flash (red), the third flash (blue) and fourth flash (green). For a detailed analysis of the situation in PsbA3-PSII see Nilsson et al. (2014) and Sugiura et al. (2018). In the PsbA3/R323E-PSII, the proton release kinetics were also very similar to those in PsbA3-PSII (Sugiura et al. 2018) although for the data point at $t = 5 \mu\text{s}$ the $\Delta I/I$ was systematically found slightly smaller on all flashes (see the discussion section).

Thirdly, the time-resolved flash dependence of the electrochromic signal was measured. The kinetics shown in Fig. 7C were recorded after the first flash (black), the second flash (red), the third flash (blue) and fourth flash (green). Comparison of the data in Fig. 7C with those in PsbA3-PSII (Boussac et al. 2020) shows that in the PsbA3/R323E mutant the kinetics were also hardly affected when compared to PsbA3-PSII, although very slightly slower in the 10 μs time-range.

In conclusion, the kinetics data reported above are unable to explain the TL properties of the mutant. Therefore, in the following, we have recorded the split EPR signals induced at cryogenic temperatures and originating from the magnetic interaction between Tyr_Z^\bullet and the Mn_4CaO_5 cluster in the S_0 , S_1 and S_2 states. Indeed, in the PsbA3/P173M mutant, which exhibits TL properties modified in the same manner as those of the PsbA3/R173E mutant,

these split signals were found significantly affected due to modifications in the geometry and/or the environment of the Tyr_Z/His190 (phenol/imidazole) motif (Sugiura et al. 2014b).

Firstly, the EPR spectrum of the S₂ state was recorded at 8.6 K after one flash given at room temperature (black spectrum in Panel A of Fig. 8). The overall spectrum, including the S₂ multiline signal, is very similar to the spectrum recorded in PsbA3-PSII under the same conditions (see e.g. Boussac et al. 2010 for a detailed description of the spectrum). The red spectrum in Panel B of Fig. 8 was recorded on the same 1-flash sample at 4.2 K. Then, the sample was submitted to a further white light illumination at 198 K and the blue spectra were recorded at 8.6 K in Panel A and 4.2 K in Panel B. In the blue spectra, two additional species were detected: the Q_A⁻Fe²⁺Q_B⁻ spectrum at $g = 1.6$ (~ 4600 gauss; see Boussac et al. 2010 and references therein) and the S₂Tyr_Z[•] split signal centered at $g = 2$ (~ 3400 gauss; see Havelius et al. 2012, Boussac et al. 2007, Petrouleas et al. 2005). The difference spectrum blue-*minus*-red is shown in Panel A of Fig. 9 with an expanded magnetic field scale (red spectrum). The black spectrum was obtained by the same way in PsbA3-PSII and is shown for comparison. The two spectra have been arbitrarily normalized to approximately the same amplitude and since the yield of formation of the split signal in the PsbA3/R323E mutant appeared much smaller this resulted in a spectrum with a lower signal-to-noise ratio. As in PsbA3-PSII, the split signal induced by visible light illumination in S₂ is not stable at 4.2 K but can be induced again by a further illumination at 4.2 K. The other important point here is that the shape of the S₂Tyr_Z[•] split signal in PsbA3/R323E-PSII significantly differed from that one in PsbA3-PSII.

Another (S₂Tyr_Z[•])' split signal can be induced by near-infrared (NIR) illumination at 4.2 K of a sample previously illuminated with two flashes at room temperature (Petrouleas et al. 2005, Boussac et al. 2007). The red spectrum in Panel B in Fig. 8 shows the spectrum at 4.2 K of the PsbA3/R323E-PSII after such a two flash-illumination and the blue spectrum was recorded after a further illumination at 813 nm at 4.2 K. The difference spectrum blue-*minus*-red is shown in Panel B of Fig. 9 with an expanded magnetic field scale (red spectrum). The black spectrum was obtained by the same way in PsbA3-PSII and is shown for comparison. The two spectra have been arbitrarily normalized to approximately the same amplitude. The NIR-induced (S₂Tyr_Z[•])' split signal in PsbA3/R323E-PSII also significantly differed from that one in PsbA3-PSII.

The red spectrum in Panel C of Fig. 8 was recorded at 4.2 K after 3 flashes given at room temperature in the presence of PPBQ in PsbA3/R323E-PSII in which the majority of centers are in the S₀ state. The S₀ multiline signal between 2400 and 4500 gauss is clearly

detectable (Messinger et al. 1997, Åhrling et al. 1998, Boussac et al. 2010). Due to the presence of PPBQ, the oxidized non-heme iron signal between 700 and 1200 gauss is also present (Zimmermann and Rutherford 1986). The blue spectrum was recorded after a further continuous white light illumination given to the sample in the EPR cavity at 4.2 K. In addition to the disappearance of the non-heme iron signal a split signal centered at $g = 2$ was also light-induced. The difference spectrum blue-*minus*-red is shown in Panel C in Fig. 9 with an expanded magnetic field scale (red spectrum). The black spectrum was obtained by the same procedure in PsbA3-PSII and is shown for comparison. Again, the $S_0\text{Tyr}_Z^\bullet$ split signal in PsbA3/R323E-PSII significantly differed from that one in PsbA3-PSII.

Finally, the red spectrum in Panel D of Fig. 9 shows the $S_1\text{Tyr}_Z^\bullet$ split signal in PsbA3/R323E-PSII upon visible light illumination at 4.2 K and the black spectrum corresponds to the $S_1\text{Tyr}_Z^\bullet$ split signal in PsbA3-PSII. This split signal also differed in the two samples.

In conclusion, all the split EPR signals induced in PsbA3/R323E-PSII differed from their equivalents in PsbA3-PSII. In addition, although it is difficult to precisely estimate their yield of formation, these signals clearly appeared to be induced in a smaller proportion of centers in the mutant than in the wild type. Modified split signals formed with a lower efficiency are two observations already done in the PsbA2-PSII and PsbA3/P173M-PSII, in which the geometry and or the environment of Tyr_Z likely differs from that in PsbA3-PSII. In these PSII samples, the P_{680}^+ decay was also found to be significantly slowed-down in the tens of μs time range where the proton transfer(s) coupled to the electron transfer take(s) place (Sugiura et al. 2014b). In contrast, the decay in the tens of ns time domain, corresponding to the pure electron transfer between Tyr_Z and P_{680}^+ , was not affected. We therefore asked the question as to whether the R323E site-directed mutation also determines the kinetics specificity of the oxidation of Tyr_Z by P_{680}^+ .

Panel A in Fig. 10 shows the S_n -state dependent P_{680}^+ reduction rate. The transient flash-induced absorption changes at 432 nm after the first flash (black symbols), second flash (red symbols), third flash (blue symbols) and fourth flash (green symbols) given to dark-adapted PsbA3/R323E-PSII have been recorded from 5 ns to 100 ms after each of the flashes. In this spectral region, the redox changes of several species, such as the chlorophylls, cytochromes, Tyr_Z , and Q_A , may contribute to the absorption profile. However, the largest spectral contribution upon flash illumination in the Soret region of the chlorophyll is that one associated with the bleaching of P_{680} upon its oxidation which peaks at 432 nm, for example

(Diner et al. 2001). The $\Delta I/I$ was slightly less negative in the 5 ns to 100 ns time domain after the 2nd and 3rd flash showing that the pure electron transfer from Tyr_Z to P₆₈₀⁺ was slightly slower in the S₂ and S₃ states than in the S₀ and S₁ states, as in PsbA3-PSII (e.g. Sugiura et al. 2014b). Panel B of Fig. 10 shows an average (to improve the signal-to-noise ratio) of the $\Delta I/I$ recorded at 20 ns, 40 ns and 80 ns plotted versus the flash number in PsbA3/R323E-PSII (red trace) and PsbA3-PSII (black trace). The amplitudes of the oscillations with a period of four showing the larger $\Delta I/I$ on the 2nd and 3rd flashes were similar in PsbA3/R323E-PSII and PsbA3-PSII as this was observed above with other spectroscopic probes (Fig. 4). However, the average value of the $\Delta I/I$ in Panel B of Fig. 10 was more negative in PsbA3/R323E-PSII than in PsbA3-PSII. This means that the decay of P₆₈₀⁺ was slower in the mutant in this time range. This slowdown is differently illustrated in Panels A to D in Fig. 11 in which the P₆₈₀⁺ decay kinetics is shown after the 1st, 2nd, 3rd and 4th flash, respectively, in PsbA3/R323E-PSII (red data points) and PsbA3-PSII (black data points). In contrast to the situation in the PsbA3/P173M, in which the P₆₈₀⁺ decay kinetics were slowed down in the tens of μ s time range (Sugiura et al. 2014b), the R323E mutation mainly affects the P₆₈₀⁺ decay kinetics in the hundreds of ns time range. This is particularly observable after the 1st flash and 4th flash where the global P₆₈₀⁺ decay is the fastest, thus allowing an easier detection of the decay in the 10 to 20 ns time range. This time domain corresponds to the pure electron transfer from Tyr_Z to P₆₈₀⁺ and it seems not affected in the PsbA3/R323E mutant, as this was also the case in the PsbA3/P173M mutant.

Discussion

The PsbA3/R323 amino acid together with other charged residues, including PsbA3/H304 and carboxyl terminus of PsbV/Y137, surround an hydrogen bond network possibly linking the Mn₄CaO₅ cluster to the lumen through Tyr_Z (Umena et al. 2011; Fig. 1). It has been proposed that these residues could contribute to regulate a proton egress pathway via this channel (Umena et al. 2011). In order to test this suggestion, a PsbA3/R323E site-directed mutant has been constructed and characterized.

Neither the experiments probing the oscillations with a period four, characteristic of the redox transitions in the Mn₄CaO₅ cluster under a flash sequence, nor those probing the stoichiometry of the proton releases occurring in the different S-state transitions were affected by the PsbA3/R323E mutation. The capability of Mn₄CaO₅ cluster to normally function is therefore not altered by the mutation. In contrast to these lacks of effects, other experiments revealed important differences in the PsbA3/R323E-PSII mutant when compared to the

PsbA3-PSII. Those are (1) the energetics of the $S_2Q_A^-/DCMU$ and $S_3Q_A^-/DCMU$ radiative charge recombination; (2) the $S_0Tyr_Z^\bullet$, $S_1Tyr_Z^\bullet$, $S_2Tyr_Z^\bullet$ split EPR signals induced at 4.2 K by visible light from the S_0Tyr_Z , S_1Tyr_Z , S_2Tyr_Z , respectively, and the $(S_2Tyr_Z^\bullet)'$ induced by NIR illumination at 4.2 K of the S_3Tyr_Z state; (3) the decay kinetics of P_{680}^+ in the hundreds of ns time range. In addition, we may add the slight changes in the μs time domain of the kinetics in the spectral response of bromocresol purple and in the electrochromism of P_{D1} under a flash sequence.

Tyr_Z is the species whose changes in the properties could explain all the observations listed above in the PsbA3/R323E site-directed mutant. Firstly, the slowdown of the P_{680}^+ decay occurs in a time domain proposed to correspond to the establishment of the $Tyr_ZP_{680}^+ \leftrightarrow [Tyr_Z^{ox}P_{680}]_{initial}$ after the pure electron transfer between Tyr_Z and P_{680}^+ in the tens of ns time range and before the proton movements resulting from the oxidation of Tyr_Z ; these proton movements occurring on a large scale in the μs time domain. This equilibrium has been considered as a dielectric relaxation (Renger 2012, Klauss et al. 2012) and it would stabilize the Tyr_Z^{ox} initially formed by lowering its free-energy level (Klauss et al. 2012). In the PsbA3/R323E mutant, the slowdown of the $Tyr_ZP_{680}^+ \leftrightarrow [Tyr_Z^{ox}P_{680}]_{initial}$ equilibrium would therefore correspond to an increase of the free energy level of the $[Tyr_Z^{ox}P_{680}]_{initial}$ state. Since in the mutant the slowdown of the P_{680}^+ decay is observed on all flashes, the relaxation involved here occurs in all the $S_nTyr_Z^{ox}$ states although it seems more pronounced after the first and fourth flashes, i.e. in the $S_0Tyr_Z^{ox}$ and $S_1Tyr_Z^{ox}$ states.

Because neither the Mn_4CaO_5 cluster nor Q_A seem affected by the mutation, the TL data in the PsbA3/R323E-PSII mutant strongly suggest that this is the free energy level of one of the intermediates between either S_2 or S_3 and the luminescent state $*Chl_{D1}$ which would have a higher free energy level. So, it seems likely that this intermediate with a higher energy level is Tyr_Z^{ox} . From the empirical relationship between the shift in free energy and in the peak temperature (Cser and Vass 2007, Rappaport and Lavergne 2009), the shift in the E_m of the Tyr_Z^{ox}/Tyr_Z couple involved in the recombination process could be $\sim +20$ meV, i.e. a relatively large value when compared to the estimated energy difference, 20-30 meV, between the $[Tyr_Z^{ox}P_{680}]_{initial}$ and $[Tyr_Z^{ox}P_{680}]_{relaxed}$ states (Renger 2012). However, the similar oscillations in the mutant than in the wild type shows that this does not increase the miss parameter to a level that is detectable in the experiments reported here.

Since the $S_0Tyr_Z^\bullet$, $S_1Tyr_Z^\bullet$, $S_2Tyr_Z^\bullet$ and $(S_2Tyr_Z^\bullet)'$ split EPR signals are induced at 4.2 K, the spectral changes observed in the PsbA3/R323E mutant indicate that the structural

modifications are likely already present in the S_0 , S_1 , S_2 and S_3 states prior to the oxidation of Tyr_Z. This is in agreement with the P_{680}^+ decay affected in all the S-state transitions.

Although the R323 residue is close to a potential proton channel, there is no evidence in this work that the proton release kinetics are affected in the PsbA3/R323E mutant. However, the data in Panel B of Fig. 7 shows that for the shortest time investigated (i.e. 5 μ s), the absorption of bromocresol purple was slightly smaller on all flashes than at 10 μ s. This difference between 5 μ s and 10 μ s, not seen in PsbA3-PSII, could correspond to electrostatically triggered shift(s) of p*K* of amino acids close to the bulk (Haumann and Junge 1994) that would be induced by the slower formation of [Tyr_Z^{ox}]_{relaxed} in the mutant than in the wild type PSII.

In conclusion, the present work shows that the R323 residue of the D1 protein interacts with Tyr_Z. This interaction likely occurs via the H-bond network previously proposed to be a proton channel. However, rather than participating in the egress of protons to the bulk, this channel could be involved in the relaxations of the H-bonds around Tyr_Z thus decreasing the driving force required for its oxidation.

Author contributions

MS and AB designed research, made some of the experiments, analyzed the data and wrote the paper; JS performed the time resolved absorption experiments; TT and MN constructed the PsbA3/R323E mutant and discussed the data; NT did some PSII preparations and biochemical analysis.

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Legend of the figures

Fig. 1. (A) Amino acid residues forming the proton channel defined by Umena et al. (2011). Water molecules drawn in gray, pale green and pale orange are in D1, CP43, and PsbV, respectively. D1/R323 and D1/H304 make hydrogen bonds to carboxyl group of C-terminus of PsbV/Y137. (B) Structure of PsbV/Y137, D1/H304 and D1/R323. Both D1/H304 and D1/R323 are hydrogen-bonded to the carboxyl terminus of PsbV/Y137. The figures were drawn with MacPyMOL with the A monomer in PDB 4UB6.

Fig. 2. Construction of the PsbA3/R323E site-directed mutant. (A) Nucleotide sequence of *psbA₃* and the amino acid sequences around R323 in the WT*3 strain and the PsbA3/R323E mutant. Positions corresponding to R323 are indicated with red letters. Numbers correspond to the positions from the initial codon. Substituted nucleotides for R323E in the mutant are indicated in small letters. For making and selecting the mutant, the *AseI* restriction site (letters in italic) was created in *psbA₃-Arg323* in the same time. (B) Physical map around *psbA₃*, and theoretical length of amplified DNA by PCR using Forward (*Fw*) and Reverse (*Rv*) primers. The created *AseI* (shown as *AseI**) site for R323E mutant is at position +961. The sizes of 391 bp, 257 bp +134 bp are DNA fragments after digestion of the amplified DNA with *AseI*. (C) Agarose gel (2%) electrophoresis of PCR products using the *Fw* and *Rv* primers (lanes 2 and 4) and the DNA fragments of the products after digestion with *AseI* (lanes 3 and 5). Lane 1 and 6, 100 bp DNA ladder markers (Nacalai, Japan); lanes 2 and 3, WT*3 strain; lanes 4 and 5, R323E strain.

Fig. 3. Relative oxygen-evolving activities at different pH values of PsbA3 thylakoids (black) and PsbA3/R323E thylakoids (red). Absolute values are $330 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{h}^{-1}$ and $310 \mu\text{mol O}_2 (\text{mg Chl})^{-1} \text{h}^{-1}$ in PsbA3 thylakoids at pH 7.0 and in PsbA3/R323E thylakoids, respectively. The values of the activities are the average of at least 3 measurements

Fig. 4. Sequence of the amplitude of the absorption changes using a series of saturating flashes (spaced 300 ms apart) and given to PsbA3-PSII (black) and PsbA3/R323E-PSII (red). The samples ($\text{Chl} = 25 \mu\text{g mL}^{-1}$) were dark-adapted for 1 h at room temperature before the addition of $100 \mu\text{M}$ PPBQ dissolved in dimethylsulfoxide and $100 \mu\text{M}$ ferricyanide in Panel B. Panel A: The $\Delta I/I$ were measured at 291 nm 100 ms after each flash. Panel B: The $\Delta I/I$ were measured at 575 nm 10 ms after each flash in the presence of $150 \mu\text{M}$ bromocresol purple. Panel C: amplitude of the absorption change differences “440 nm-*minus*-424 nm” measured 100 ms after each flash.

Fig. 5. Thermoluminescence glow curves from $\text{S}_2\text{Q}_\text{A}^-/\text{DCMU}$ charge recombination measured after one flash given at -10°C followed by a dark period of 30 s before the start of the heating ramp. Black curve, PsbA3-PSII; red curve, PsbA3/R323E-PSII; blue curve, PsbA3/E130Q-PSII; green curve, PsbA3/P173M-PSII. The samples ($10 \mu\text{g Chl mL}^{-1}$) were previously dark-adapted at room temperature for at least 1 h before the addition of DCMU dissolved in

ethanol. Then the samples were loaded into the cuvette in total darkness and illuminated. The heating rate was $0.4^{\circ}\text{C s}^{-1}$.

Fig. 6. Thermoluminescence glow curves from $S_2Q_B^-$ (A) and $S_3Q_B^-$ (B), charge recombinations measured after one flash given at -10°C followed by a dark period of 30 s before the start of the heating ramp. (C-D), thermoluminescence glow curves from $S_2Q_A^-$ /DCMU and $S_3Q_A^-$ /DCMU, respectively, when the DCMU was added immediately after illumination by one or two flashes before the start of the heating ramp. Black curve, PsbA3-PSII; red curve, PsbA3/R323E-PSII; blue curve, PsbA3/E130Q-PSII; green curve, PsbA3/P173M-PSII. The samples ($10 \mu\text{g Chl mL}^{-1}$) were previously dark-adapted at room temperature for at least 1 h before to be loaded into the cuvette in total darkness. The stock solution of DCMU was in ethanol. The heating rate was $0.4^{\circ}\text{C s}^{-1}$.

Fig. 7. Time-courses of the absorption changes at 291 nm (A), of the absorption changes at 575 nm in the presence of bromocresol purple (B) and of the differences 440 nm-*minus*-424 nm (C). Measurements were done after the first flash (black), the second flash (red), the third flash (blue), and the fourth flash (green) given to dark-adapted PsbA3/R323E-PSII in the presence of $100 \mu\text{M}$ PPBQ (and $100 \mu\text{M}$ for the proton release experiment) with flashes spaced 300 ms apart. Chl concentration $A_{673} = 1.75$, i.e. $\sim 10 \mu\text{g Chl mL}^{-1}$.

Fig. 8. EPR spectra recorded in PsbA3/R323E-PSII. In Panel A, the spectra were recorded after 1 flash given at room temperature either at 8.6 K (black spectrum) or at 4.2 K (red spectrum). The blue spectrum was recorded at 4.2 K after a further white light illumination at 198 K. In Panel B, the red spectrum was recorded at 4.2 K after 2 flashes given at room temperature and the blue spectrum was recorded after a further illumination at 4.2 K with NIR light. In Panel C, the red spectrum was recorded at 4.2 K after 3 flashes given at room temperature and the blue spectrum was recorded at 4.2 K after a further white light illumination given at 4.2 K. In each Panel the spectra have been arbitrarily scaled. Instrument settings: modulation amplitude, 25 G; microwave power, 20 mW; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz.

Fig. 9. Difference EPR spectra recorded in PsbA3/R323E-PSII (red spectra) and PsbA3-PSII (black spectra) induced as indicated in Fig. 8 and in the text. Instrument settings: modulation

amplitude, 10 G; microwave power, 20 mW; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz.

Fig. 10. (A) Kinetics of the P_{680}^+ decay measured at 432 nm in PsbA3/R323E-PSII after the 1st flash (black symbols), the 2nd flash (red symbols), the 3rd flash (blue symbols) and the 4th flash (green symbols). (B) Averages of the $\Delta I/I$ measured at 20 ns, 40 ns and 80 ns in PsbA3-PSII (black) and PsbA3/R323E-PSII (red). The $\Delta I/I$ have been normalized to $A_{673} = 1.75$ for the two samples.

Fig. 11. Kinetics of the P_{680}^+ decay measured at 432 nm in PsbA3/R323E-PSII (red symbols) and PsbA3-PSII (black symbols) after the 1st flash (A), the 2nd flash (B), the 3rd flash (C) and the 4th flash (D).