Low-temperature photochemistry in photosystem II from *Thermosynechococcus elongatus* induced by visible and near-infrared light

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The active site for water oxidation in photosystem II (PSII) consists of a Mn₄Ca cluster close to a redox-active tyrosine residue (TyrZ). The enzyme cycles through five sequential oxidation states (S₀ to S₄) in the water oxidation process. Earlier electron paramagnetic resonance (EPR) work showed that metalloradical states, probably arising from the Mn₄ cluster interacting with TyrZ[•], can be trapped by illumination of the S₀, S₁ and S₂ states at cryogenic temperatures. The EPR signals reported were attributed to S₀TyrZ[•], S₁TyrZ[•] and S₂TyrZ[•], respectively. The equivalent states were examined here by EPR in PSII isolated from *Thermosynechococcus elongatus* with either Sr or Ca associated with the Mn₄ cluster. In order to avoid spectral contributions from the second tyrosyl radical, TyrD[•], PSII was used in which Tyr160 of D2 was replaced by phenylalanine. We report that the metalloradical signals attributed to TyrZ[•] interacting with the Mn cluster in S₀, S₁, S₂ and also probably the S₃ states are all affected by the presence of Sr. Ca/Sr exchange also affects the non-haem iron which is situated approximately 44 Å units away from the Ca site. This could relate to the earlier reported modulation of the potential of Q_A by the occupancy of the Ca site. It is also shown that in the S₃ state both visible and near-infrared light are able to induce a similar Mn photochemistry.

Keywords: photosystem II; oxygen evolution; electron paramagnetic resonance; Mn photochemistry

1. INTRODUCTION

Photosystem II (PSII), the enzyme responsible for oxygen evolution, catalyses light-driven water oxidation using a Mn₄Ca cluster that acts as both a device for accumulating oxidizing equivalents and the active site. The structure and ligand environment of the Mn₄Ca cluster is still unclear despite recent advances in X-ray crystallography (Ferreira *et al.* 2004; Loll *et al.* 2005) and in Mn-X-ray absorption fine structure (EXAFS) (Yano *et al.* 2006; Pushkar *et al.* 2007). Absorption of a photon by PSII induces a charge separation that results in the oxidation of a chlorophyll molecule (P₆₈₀) and the reduction of a pheophytin molecule. The pheophytin anion transfers the electron to a quinone, Q_A, and P₆₈₀⁺ oxidizes a tyrosine residue of the D1 polypeptide, tyrosine residue (TyrZ), which in turn oxidizes the Mn₄Ca cluster and the associated substrate water. During the enzyme cycle, the oxidizing side of PSII goes through five sequential redox states, denoted S_n where *n* varies from 0 to 4. Oxygen is released during the S₃ to S₀ transition in which S₄ is a transient state (Debus 2001; Diner 2001; Goussias *et al.* 2002; Britt *et al.* 2004; McEvoy & Brudvig 2004; Rutherford & Boussac 2004; Sauer & Yachandra 2004).

When either of the essential ions Ca²⁺ or Cl[−] is removed from its site, manganese oxidation can still take place, allowing the formation of the S₂ state, but in the following step, the normal S₃ state is not formed. Instead, an alternative abnormally stable form appears to be induced (Boussac *et al.* 1989). This state is thought to be the one in which the metal cluster is in the same redox state as it was in S₂ state but in addition a nearby radical is also present (Boussac *et al.* 1989). This radical is proposed to be the tyrosine residue TyrZ (e.g. Tang *et al.* 1996; Un *et al.* 2007; see also Mino & Itoh 2005). In this state, the Mn₄Ca cluster (S = 1/2) and TyrZ[•] (S = 1/2) magnetically interact, giving rise to a characteristic electron paramagnetic resonance (EPR) signal that is known as the split signal. More recently, several other ‘split’ signals were also observed in functional PSII. They were generated by illuminating PSII in the S₀, S₁ or S₂ states with visible light or in the S₃ state with near-infrared (NIR) light either at liquid helium temperatures or at 200 K. The species that were formed under these conditions were proposed to correspond to ‘non-relaxed’ S₀TyrZ[•], S₁TyrZ[•] and S₂TyrZ[•] states (Ioannidis *et al.* 2002; Koulougliotis *et al.* 2003, 2004; Zhang & Styring 2003; Zhang *et al.* 2004; Petrouleas *et al.* 2005; Havelius *et al.* 2006; Ioannidis *et al.* 2006) that could correspond to intermediates in the oxygen-evolving process.

It has been shown that the biosynthetic replacement of Ca²⁺ by Sr²⁺ in the thermophilic cyanobacterium *Thermosynechococcus elongatus* had multiple effects on

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Figure 1. Split EPR spectra in D2-Y160F-PSII with either Ca (black spectra) or Sr (red spectra) in the Mn_4 cluster. For (a–c), the spectra were first recorded immediately after the 400–350 nm illumination (light spectra). Then, the black spectra were recorded after 2–4 min at 4.2 K. The light-minus-dark difference spectra are shown in (a) S_0 , (b) S_1 , (c) S_2 . For (d) S_3 , the dark spectra were recorded prior to the NIR illumination. Instrument settings: modulation amplitude, 10 G; microwave power, 20 mW; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz and temperature, 4.2 K. The chlorophyll concentration was 1.2 mg ml^{-1} . In each panel, spectra were roughly scaled to the same spin concentration as estimated by a double integration.

Figure 2. Effect of near-infrared light (820 nm) in S_3 in D2-Y160F-PSII. The black spectrum was recorded in S_3 and the red spectrum was recorded after an additional illumination at 820 nm at 4.2 K in the EPR cavity for 2 min. The blue spectrum is the difference spectrum after-minus-before the NIR illumination. Instrument settings: modulation amplitude, 25 G; microwave power, 20 mW; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; and temperature, 4.2 K. The chlorophyll concentration was 1.2 mg ml^{-1} .

kinetics properties of the enzyme probably arising from Sr^{2+} -induced S-state-dependent shifts in the redox properties of both the manganese complex and the TyrZ (Boussac *et al.* 2004). In this work we report fully resolved spectra from the split signals arising in

Figure 3. Formation and decay of the split signal in S_3 . Amplitude of the split signal at the field position indicated by the arrow in figure 2 was followed versus time at 4.2 K in different conditions. Black trace; the sample in S_3 was illuminated at 820 nm (upward arrow) until the light was switched off (downward arrow). Red trace; at the end of the recording of the black trace, the sample was further illuminated (upward arrow) with visible light (400–650 nm) until the light was switched off (downward arrow). The blue trace was recorded on a different S_3 sample which was illuminated with visible light (400–650 nm; upward arrow) until the light was switched off (downward arrow). Instrument settings are the same as in figure 2.

Figure 4. Effect of illumination with visible light at 180 K in S_3 in WT-PSII. Spectra in (a) were recorded at 4.2 K and spectra in (b,c) were recorded at 15 K. The black spectra were recorded in S_3 and the red spectra were recorded after an additional illumination with visible light at 180 K. The blue spectra are the difference spectra after-minus-before the 180 K illumination. Microwave frequency, 9.4 GHz; modulation frequency, 100 kHz. Other instrument settings: (a), modulation amplitude, 25 G; microwave power, 20 mW; (b), modulation amplitude, 25 G; microwave power, 5 mW; (c), modulation amplitude, 2.8 G; microwave power, 20 μ W. The central part of the spectra corresponding to the TyrD' signal was deleted.

oxygen-evolving Ca- and Sr-containing PSII isolated from *T. elongatus*. In order to obtain spectra without spectroscopic overlap from TyrD', we used the D2-Y160F mutant of PSII which was engineered to remove

TyrD' (Sugiura *et al.* 2004). In addition we report a study of the different effects of NIR and visible light in the S_3 state. Lastly and somewhat surprisingly, the replacement of Ca^{2+} by Sr^{2+} appears to influence the non-haem iron which is located on the other side of the reaction centre protein.

2. MATERIAL AND METHODS

His-tagged wild-type (WT) (Sugiura & Inoue 1999) and His-tagged D2-Y160F (Sugiura *et al.* 2004) *T. elongatus* cells were grown in the presence of either Ca or Sr salts (Boussac *et al.* 2004) and PSII were purified as previously described (Boussac *et al.* 2004) with the following modifications. The presence of glycerol prevents the detection of the split signals induced by visible light (Ioannidis *et al.* 2002; Zhang & Styring 2003; Havelius *et al.* 2006), therefore, glycerol was omitted from the elution of the PSII cores from the Ni column and in the final re-suspension medium which contained 1 M betaine, 40 mM Mes, 15 mM MgCl_2 and 15 mM CaCl_2 (the pH was adjusted with NaOH to 6.5). The PSII samples were stored in liquid nitrogen at a concentration of approximately 2 mg of Chl ml^{-1} until they were used.

Continuous wave-EPR (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 an Nd:YAG laser (532 nm, 550 mJ, 8 ns Spectra Physics GCR-230-10). PSII samples at 1.2 mg of Chl ml^{-1} were loaded under dim light into quartz EPR tubes and further dark adapted for 1 hour at room temperature. Then, the samples were synchronized in the S_1 state with one pre-flash (Styring & Rutherford 1987). After another dark period for 1 hour at room temperature, 0.5 mM PPBQ dissolved in Me_2SO was added (the final concentration of Me_2SO was approx. 2%). After illumination by 0, 1, 2 or 3 flashes to form the S_1 , S_2 , S_3 and S_0 states, respectively, the samples were frozen in the dark at 198 K in a dry ice ethanol bath, degassed at 198 K and then transferred to 77 K. NIR illumination of the samples was done directly in the EPR cavity and was provided with a laser diode emitting at 820 nm (Coherent, diode S-81-1000C) with a power of 600–700 mW at the level of the sample. Illumination of the samples with visible light was done with a 250 W projection lamp filtered with water, five calflex filters and a Corion LS-650 filter resulting in a broadband illumination from 400 to 650 nm (designated visible light hereafter). Illumination with visible light at 180–200 K of the samples was done in a nitrogen gas flow system (Bruker, B-VT-3000).

3. RESULTS AND DISCUSSION

The S_n states were generated by laser flashes illumination at room temperature in D2-Y160F PSII containing either Ca (black spectra) or Sr (red spectra). In figure 1a, PSII in the S_0 state were illuminated at 4.2 K for 1–2 s by visible light. EPR spectra were recorded immediately after this short illumination and then after dark adaptation for 2–4 min at 4.2 K. The signal that decayed during this dark period is shown in figure 1a. On the time scale required for the recording of these spectra, the small amount of $\text{Car}^{\cdot+}/\text{Chl}^{\cdot+}$ radicals generated in a fraction of centres by the low-temperature illumination is virtually stable (not shown), so that the difference spectra shown in figure 1a and attributed to $S_0\text{TyrZ}^{\cdot-}$ (Zhang & Styring

2003) are essentially without any contribution from these free radical species. The spectra in Ca- and Sr-PSII are similar but not identical. The width (low-field peak to high-field trough) varies from 130 gauss in Sr-PSII to 146 gauss in Ca-PSII. This latter value found in the cyanobacterium studied here is slightly smaller than that found in plant Ca-PSII (approx. 165 gauss; Havelius *et al.* 2006).

Figure 1*b* shows the spectra induced by an illumination with visible light at 4.2 K in the S_1 state. The protocol was identical to that described in figure 1*a*. Spectra in Ca- and Sr-PSII attributed to S_1 TyrZ' (Zhang & Styring 2003) are similar but not identical. This split signal is slightly broader in Sr-PSII (87 gauss, low-field peak to high-field trough) than in Ca-PSII (73 gauss). In plant Ca-PSII, the width was found to be slightly narrower (approx. 68 gauss; Havelius *et al.* 2006).

In figure 1*c*, the Ca-PSII sample trapped in the S_2 state was illuminated in the visible range at 200 K (note the different magnetic field scale in this figure). In plant PSII, this protocol was shown to be able to induce a split signal attributed to S_2 TyrZ' and that decays in the minutes time scale by charge recombination at 4.2 K with the electron from Q_A^- (Ioannidis *et al.* 2006). Similar behaviour occurs in Ca-PSII from *T. elongatus* (not shown). The $\text{Car}^{\cdot+}/\text{Chl}^{\cdot+}$ radicals generated in a fraction of centres by the 200 K illumination were stable at 4.2 K in the time scale where most of the split signal decayed (not shown), so that the difference spectrum shown in figure 1*c* (black spectrum) is not contaminated by these free radical species. In Sr-PSII, we found that the 200 K illumination induced only a very weak signal (not shown). Therefore, several other temperatures were tested and we found that a signal was induced with its highest amplitude (although still weaker than that in Ca-PSII) upon illumination at 180 K (N.B. in each panel of figure 1 the spectra are normalized, see the legend). A minor contribution from another free radical signal may be present in the weaker S_2 TyrZ' signal in Sr-PSII (red spectrum, figure 1*c*). Clearly, the presence of Sr in the place of Ca changes the enzyme in such a way that its temperature dependence is affected. In plant Ca-PSII, the low-field peak to high-field trough spacing was 116 gauss (Ioannidis *et al.* 2006) and is found here in *T. elongatus* to be 110 gauss in Ca-PSII and 100 gauss in Sr-PSII.

The split signals shown in figure 1*a–c* in *T. elongatus* were generated by a charge separation triggered by visible light at low temperature and are interpreted as originating from the S_0 TyrZ' (figure 1*a*), S_1 TyrZ' (figure 1*b*) and S_2 TyrZ' (figure 1*c*) states in accordance with the literature reports in plant PSII (Ioannidis *et al.* 2002; Koulougliotis *et al.* 2003; Zhang & Styring 2003; Zhang *et al.* 2004; Petrouleas *et al.* 2005; Havelius *et al.* 2006; Ioannidis *et al.* 2006). The use of the D2-Y160F mutant allowed us to detect the central part of the spectra without any additional treatment like those used in plant PSII to reduce TyrD' (Havelius *et al.* 2006).

An S_3 TyrZ' state has yet to be reported. In S_3 , NIR illumination at 4.2 K does result in the formation of a split signal (Petrouleas *et al.* 2005). However, this signal was attributed to S_2 TyrZ' formed by NIR-induced conversion of the Mn cluster into a state so

oxidizing that the back reaction, S_3 TyrZ to S_2 TyrZ', occurs (Petrouleas *et al.* 2005). The split signals generated in the S_3 state by NIR illumination in Ca-PSII (218 gauss width) and Sr-PSII (295 gauss width) are shown in figure 1*d*. The use of the D2-Y160F mutant makes the detection of these spectra possible without any contributions from any free radicals generated by the reaction centre photochemistry. The two spectra present some similarities in their structure, but clearly the signal is broader in Sr-PSII than in Ca-PSII.

The miss parameter and the S-state composition in dark adapted samples upon a pre-flash illumination have been shown to be very similar in Ca- and Sr-containing PSII (Boussac *et al.* 2004), so that a different S-state population in these two PSII samples after 0, 1, 2 and 3 flashes cannot explain the spectral differences detected in figure 1. There are several further arguments in support of the conclusion that the spectral differences between Ca- and Sr-PSII shown for each S state are real effects due to the metal exchange rather than mixing S states. (i) The S_2 split signal is light induced at a temperature much higher than that at which the S_1 split signal can be generated, so that we can exclude contributions from the S_1 split signal in the spectra of figure 1*c*. (ii) The S_3 split signal is induced by NIR illumination at 4.2 K in the sample in the S_3 state. Thus, in figure 1*d*, contributions from both S_1 and S_2 split signals, which are induced only by visible light, are unlikely. (iii) In the S_0 state, the effect of Sr exchange is a slight narrowing of the split signal (figure 1*a*). In contrast, in the S_3 state, the split signal generated (in figure 1*d* by NIR light but a similar effect is seen with visible light, see figure 5) in Sr-containing PSII is markedly broader than that in the Ca-containing PSII. Thus, contributions from the centres starting in the S_3 state (after two flashes) to the spectra shown in figure 1*a* (after three flashes) are not detectable.

We can conclude that in addition to the well-known effect of the Ca/Sr exchange on the EPR properties of the S_2 state (Boussac & Rutherford 1988; Boussac *et al.* 2004), figure 1 shows that Ca/Sr exchange also affects the magnetic properties of the Mn_4 cluster in all the S_n states, and/or the magnetic coupling between the cluster and TyrZ'. All of these split signals are different from those formed in PSII inhibited by either acetate treatment or Ca depletion and also slightly differ from those in PSII from plants. The fully resolved split signals presented here should be used in future work to characterize structural changes in the TyrZ– Mn_4 environment occurring in the different states.

NIR illumination in the S_3 state (black spectrum) produces a complex signal (red spectrum; figure 2). In addition to the split signal with a peak to trough width of 176 gauss, signals at lower field and higher field were also induced. The NIR-induced spectrum (blue spectrum) exhibits a feature which peaks at $g \approx 5.20$ (turning point at $g \approx 4.35$) and a smaller peak at $g \approx 1.51$ (Boussac *et al.* 2000). The signal at $g \approx 5.20$ is thought to correspond to a spin 7/2 state of the Mn cluster (Sanakis *et al.* 2001).

In our conditions the NIR-induced split signal was stable for at least 4 hours at 4.2 K after the NIR illumination (not shown). This result contrasts

somewhat with previous reports in which a decay of this split signal was observed (Petrouleas *et al.* 2005). This discrepancy could originate from a difference in the quality of light, i.e. visible light filtered by an RG 715 Schott filter (Petrouleas *et al.* 2005) versus 820 nm laser light used here. Alternatively, the different protocol used to generate the S_3 state could explain the different stabilities of the signal, i.e. the continuous illumination at 240 K resulting in a reduced electron acceptor side (Petrouleas *et al.* 2005) versus flash illumination at room temperature resulting in an oxidized electron acceptor side as used in this work. In figure 3 the effects of light in the NIR and visible range were therefore tested separately in the S_3 state. The black trace shows the formation of the split signal (at the magnetic field position indicated by the arrow in figure 2) under continuous illumination at 820 nm at 4.2 K. Then, following the NIR illumination, the sample was further illuminated with visible light (400–650 nm; red trace). This visible illumination induced an increase of the signal, the spectrum of which was similar to that induced by NIR light (not shown). Unlike the signal induced by NIR light, this signal was not stable at 4.2 K and decayed to the original level reached prior to the visible illumination. Each further illumination in the visible range regenerated a signal with similar amplitude and a similar decay (not shown). In a second experiment, the sample in S_3 was illuminated only with visible light (blue trace). The EPR spectrum of this signal was similar to that induced by NIR light (not shown) and as shown by the blue trace, a fraction of which decayed with kinetics comparable to that of the red trace and the rest of the signal was stable.

The experiment reported in figure 3 indicates that the Mn_4 clusters that exhibit an absorption band in the NIR range (Boussac *et al.* 2005) also exhibit an absorption band in the visible range (blue trace). In addition, some of the centres in which Mn photochemistry was not triggered by NIR light could be nevertheless triggered by visible light (red trace). Upon illumination with visible light (blue and red traces), part of the split signal decayed at 4.2 K. Two explanations can be put forward for this decay: (i) the visible light induces a charge separation in the S_3 state resulting in a $S_3X^+Q_A^-$ state (where S_3X^+ is responsible for the split signal) that would recombine into the S_3XQ_A state at 4.2 K. In this case, the S_3X^+ EPR signal would have to be coincidentally very similar to that of the S_2TyrZ' . This weighs somewhat against this hypothesis. (ii) Alternatively, the visible light induces two photochemical events in the same reaction centre: first, the conversion of S_3TyrZ to S_2TyrZ' by Mn photochemistry, where S_2TyrZ' is responsible for the split signal, and second, the formation of $S_2TyrZ'-(Car^+/Chl^+/cytb_{559}^{ox})-Q_A^-$ by chlorophyll photochemistry, which then would decay to $S_2TyrZ-(Car^+/Chl^+/Cytb_{559}^{ox})-Q_A$ at 4.2 K by a charge recombination between $TyrZ'$ and Q_A^- .

These two hypotheses were addressed in the following experiment. Samples in S_3 were pre-illuminated at 180 K with visible light to form Q_A^- and reduce the oxidized non-haem iron thus minimizing further charge separation upon additional visible light illumination at 4.2 K.

In order to better quantify the effect of these low-temperature illuminations, a normal WT PSII was used in order to benefit from the $TyrD'$ signal as a spin-counting reference.

The effects of the 180 K pre-illumination in S_3 are depicted in figure 4. The black and red spectra were recorded prior to and after the 180 K illumination, respectively. The difference spectra (after-minus-before the 180 K illumination) are in blue. Spectra were recorded at 4.2 K in figure 4a and at 15 K in figure 4b,c. Several comments can be made: (i) the non-haem iron, which was oxidized in a fraction of the centres, was reduced by the 180 K illumination (negative signals at 870 and 1182 gauss, g values = 7.7 and 5.65, respectively), (ii) the 180 K illumination generated the $Q_A^-Fe^{II}$ state (signal at 3400–3800 gauss, g value approx. 1.9) presumably in centres in which the non-haem iron was not oxidized, (iii) $cyt b_{559}$ was oxidized in a fraction (less than or equal to 40%) of centres (signals at 2213 gauss ($g_z=3.04$), 3074 gauss ($g_y=2.19$) and 4700 gauss ($g_x=1.43$)), (iv) the 180 K illumination did not affect the amplitude of the S_3 signal observed at 300–1000 gauss and centred at $g=9.4$, and (v) in figure 4c, it can be seen that the 180 K illumination induced a Car^+/Chl^+ signal in approximately 5% of the centres (assuming $TyrD$ was oxidized in all centres).

Based on the estimate of the proportion of oxidized electron donors, the number of centres containing Q_A^- after the 180 K illumination may not be 100%. However, it is possible that other species, not detected under the experimental conditions (i.e. the buffer composition) used here, may also act as electron donors. In any case, the effect of 180 K pre-illumination should significantly decrease the proportion of centres that are able to undergo a charge separation upon a subsequent illumination at 4.2 K.

Figure 5a shows an experiment in which the S_3 sample was illuminated by visible light at 4.2 K immediately after the 180 K illumination (red spectrum). The amount of the split signal generated under these conditions is comparable to (although systematically slightly larger than, not shown) that in the absence of the 180 K pre-illumination procedure (figures 2 and 3). This confirms that formation of the split signal under illumination with visible light occurred independently of the charge separation process. Upon dark adaptation for 4 hours at 4.2 K (blue spectrum), approximately 50% of the split signal decayed. This decay was accompanied by a decrease in the $Q_A^-Fe^{II}$ signal and this is consistent with a charge recombination process, i.e. the hypothesis (ii) stated above.

Unfortunately while hypothesis (ii) may occur, an additional experiment showed that the situation is not so simple. When an additional NIR illumination at 820 nm was given after the recording of the blue spectrum, i.e. when approximately 50% of the S_2TyrZ' state had disappeared, the split signal was fully regenerated (green spectrum). From hypothesis (ii) the centres were expected to be in the $S_2TyrZ-(Car^+/Chl^+/cytb_{559}^{ox})-Q_A$ state after the 4 hours incubation at 4.2 K, therefore it is difficult to understand how the NIR illumination could reform the split signal (i.e. S_2TyrZ') from the S_2TyrZ state. We can tentatively put forward the following hypothesis. It is well established that the spin state of

Figure 5. Effect of illumination with visible and NIR light in S_3 , in WT PSII, in samples pre-illuminated at 180 K. In (a), the S_3 sample illuminated at 180 K with visible light was further illuminated with visible light at 4.2 K (red spectrum). Then blue spectrum was recorded after a dark period of 4 hours at 4.2 K. Green spectrum was then recorded after a further NIR illumination for 2 min at 4.2 K. In (b), the S_3 sample illuminated at 180 K with visible light was illuminated with visible NIR light at 4.2 K (red spectrum). Then blue spectrum was recorded after a dark period of 2 hours at 4.2 K. Green spectrum was then recorded after another NIR illumination for 2 min at 4.2 K and the black spectrum after a further illumination with visible light. Instrument settings are same as in figure 2. The central part of the spectra corresponding to the TyrD' signal was deleted. The insets show an expanded view of the split signal magnetic field region.

the Mn_4 cluster in S_2 is strongly affected by NIR illumination (Boussac *et al.* 2005 and references therein). Moreover, it was reported that NIR illumination at 5 K of the S_2 state could probably induce an S_2 TyrZ to S_1 TyrZ' back reaction (Kouloughiotis *et al.* 2003) in a small proportion of centres. Therefore, we cannot exclude that upon NIR illumination, S_1 TyrZ' could be formed in a fraction of the centres that decayed into the S_2 TyrZ state during the dark period at 4.2 K (figure 5a). If so, the split signal formed in these conditions would have to be identical to that originating from S_2 TyrZ' upon NIR illumination of the S_3 TyrZ state, and this seems rather unlikely.

In the experiment reported in figure 5b, the S_3 sample was illuminated by NIR light (red spectrum) at

Figure 6. EPR spectra of the non-haem iron. Spectra a and c were recorded in the S_2 state of Ca and Sr containing PSII, respectively. Spectra b and d were recorded after a further 200 K illumination in Ca and Sr containing PSII, respectively. The difference spectra after-minus-before the 200 K illumination are shown in the centre part of the panel. Instrument settings: modulation amplitude, 25 G; microwave power, 20 mW; microwave frequency, 9.4 GHz; modulation frequency, 100 kHz; temperature, 8.5 K. The chlorophyll concentration was 1.2 mg ml^{-1} .

4.2 K immediately after the 180 K pre-illumination procedure. Again a large split signal was observed (this NIR-induced split signal is also slightly larger here than that produced in the conditions of figure 2). More than half of the NIR-induced split signal disappeared in 2 hours at 4.2 K (blue spectrum) together with a decay of the $Q_A^- \text{Fe}^{II}$ signal. A second NIR illumination at 4.2 K almost completely regenerated the split signal (green spectrum). Then, an additional visible illumination increased the split signal (black spectrum).

From the results in figure 5 it appears that a pre-illumination with visible light of an S_3 sample at 180 K closed a high proportion of centres by reducing the electron acceptors, but does not prevent the formation of the split signal generated at 4.2 K by both visible and NIR illumination. Moreover, the amplitude of the split signals generated at 4.2 K is systematically larger than that induced without the 180 K pre-illumination. The origin of this effect remains to be explained. It is probably not an influence of the positive charges ($\text{Car}^+/\text{Chl}^+$) generated by the 180 K illumination because the proportion of centres in which these radicals were formed is small. Quantification with a field-swept electron spin-echo experiment of the split signal induced by NIR in the S_3 state done as previously described for Ca^{2+} -depleted PSII (Boussac 1996) indicates that it is formed in 20–30% of the centres (not shown). Visible light at 180 K seems to increase

slightly this proportion by triggering a photochemical event in/or around the Mn₄ cluster. Further experiments will be required to test these hypotheses and it seems probable that some of the structural heterogeneities at the origin of the different photosynthetic electron transfer process at low temperature could also affect the Mn photochemistry triggered by NIR and/or visible lights.

It has been proposed that the photoinhibition process of PSII is triggered in part by the absorption of blue light by the Mn₄ cluster (Vass *et al.* 2002; Ohnishi *et al.* 2005; Sarvikas *et al.* 2006). It is possible that this phenomenon and the effects of visible light described in this work reflect the same Mn photochemistry.

During the course of this work, we observed that the non-haem iron signal slightly differed in Ca- and Sr-containing D2-Y160F PSII, which is shown in figure 6. The red spectrum (a, Ca sample) and blue spectrum (c, Sr sample) were recorded in the S₂ state formed by one flash at room temperature in the presence of PPBQ. The PPBQ semiquinone formed on the flash oxidizes the non-haem iron in a large fraction of the centres (Zimmermann & Rutherford 1986). Then the Ca and Sr samples were further illuminated at 200 K (black spectra b and d). This resulted in the reduction of Fe^{III} to Fe^{II} by the Q_A⁻ formed under the illumination. The difference spectra show that the non-haem iron environment differs slightly in these two samples (e.g. a shift from 7.7 to 7.5 for the *g* value of one of the resonances). It was recently reported that the electron transfer from Q_A⁻ to Q_B was slowed down in Sr-PSII (Kargul *et al.* 2007). Such a slowdown could be the consequence of the structural change around the non-haem iron observed here. Since this Sr-PSII preparation contains only one Sr per PSII (Boussac *et al.* 2004), this observation appears to reflect a long-distant effect between the specific Ca site in the oxygen-evolving complex and the PSII electron acceptor side that may be related to the change of the midpoint potential of Q_A observed when Ca²⁺ is removed from PSII (Krieger & Rutherford 1997). Such a change on the acceptor side is the second long-distant effect upon Ca/Sr exchange which has been already observed. The first one was a lower affinity of the extrinsic proteins that could be countered by the addition of betaine (Boussac *et al.* 2004).

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Discussion

Boussac Q & A edited by J. Barber

F. Ho (*Uppsala University, Uppsala, Sweden*). How definitive is your assignment of Mn1 as the Mn(III) ion in the S_2 state? To which extent would it affect spectral simulations and exchange coupling schemes?

A. Boussac. In order to reproduce the isotopic hyperfine coupling obtained from X-band EPR simulations, we placed the Mn(III) in different positions compared to J. Messinger in his talk. The location shown is favoured on the basis of the J-values and the possibility to explain other EPR signals (such as $g=4.1$).