**Supplementary Materials for:**

*Multiple Redox-Active Chlorophylls in the Secondary Electron-Transfer Pathways of Oxygen-Evolving Photosystem II*

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**EPR Spectra of O₂-Evolving PS II**

The EPR spectra of O₂-evolving PS II samples used to generate g-values for Figure 8 are shown in Figure S1. The EPR signals were generated with white light from a halogen bulb controlled by a shutter, the same method as for the near-IR spectra. EPR conditions are given in the Figure legend. Based on the g-value (Figure 8A) and line shape (Figures S1A and 8B), the initial EPR signal is primarily from a tyrosine radical (oxidized Y$_D$). Spectra measured after longer illumination times are primarily from Chl and Car radicals.

**Near-IR spectra of Mn-depleted PS II depleted by ascorbate**

Photosystem II core complexes were Mn-depleted by incubation with 4 mM ascorbate for 5 hours at 4 °C in the dark. Ascorbate also reduces Cyt $b_{559}$; therefore, following centrifugation and resuspension to wash out the released manganese, the sample was treated with ferricyanide to oxidize the heme. The PS II sample was prepared in a glycerol buffer, as described in the text, and frozen to 20 K. A near-IR spectrum was collected of the sample prior to illumination, then the sample was illuminated for 15 min and a second spectrum was collected. The light-minus-dark difference spectrum is shown in Fig S2. The spectrum appears similar to the spectrum collected for a Mn-depleted PS II sample that was Mn-depleted by hydroxylamine treatment.
**Oxidation of Chl and Car at High pH**

It has been reported that Y_D oxidation is rapid at high pH (t_1/2 ~ 190 ns, pH = 8.5) (1). To test whether Y_D competes with Car and Chl as a donor to P_680^+, we measured carotenoid and chlorophyll photooxidation in PS II samples at high pH. For studies of PS II over the pH range from 6.0 to 8.5, buffer was prepared with 25 mM MES, 25 mM 1,4-bis(4-sulphobutyl)piperazine (PIPBS), 20 mM CaCl_2, 5 mM MgCl_2, 63% (v/v) glycerol, and the pH was adjusted with NaOH to the specified pH value.

The samples, ranging between pH 6.0 and pH 8.5, were treated with 5 mM ferricyanide, frozen, illuminated for 15 min at 20 K and the near-IR light-minus-dark difference spectra are shown in Figure S3. Clearly, the total yield of Car^+ and Chl^+ decreased at higher pH. But, it is possible that this decrease is due to either fewer stable charge separations formed in PS II at high pH values or an increase in the electron donation from another donor such as reduced Cyt b_{559} or Y_D. Because of the concentration of PS II used here, we could monitor redox changes in Q_A, Car, Chl, and Cyt b_{559} in the same experiment. We measured Q_A reduction by the appearance of the C550 feature after low temperature illumination and found that this value is constant for all pH values, except for a slight decrease at the highest pH values (data not shown). Because the level of Q_A^- does not decrease significantly at high pH, the lower yield of Car^+ and Chl^+ implies another species is oxidized by P_680^+ at high pH. As mentioned above, it is known that electron transfer from Y_D to P_680^+ occurs at high pH and will compete with Car and Chl oxidation. Therefore, if Y_D were already oxidized in the sample at high pH, Car and Chl should again be oxidized. To test this hypothesis, we pre-oxidized Y_D at room temperature by the illumination procedure described in the Methods. Because Cyt b_{559} can be photoreduced by the procedure to make oxidized Y_D, we added additional ferricyanide to the sample after room temperature illumination. Under these conditions, where Y_D and Cyt b_{559} are oxidized, then Car and Chl photooxidation is observed at high pH similar to the yield at pH = 6.0 (see Figure S3C). Therefore, the lower yield of Car and Chl in PS II at higher pH is due to a competing donor, tyrosine D.

**Temperature Dependence of Chl^+ Formation**

We investigated the temperature dependence of the near-IR absorption signals formed in O_2-evolving *Synechocystis* PS II core complexes. Samples were illuminated by white light for 15 min and the light-minus-dark difference spectra at each temperature, 20 K, 85 K, 120 K, 140 K
and 160 K, are shown in Figure S4. The absorbance maximum of the Car$^+$ shifts to longer wavelengths at warmer illumination temperatures (see Figure S4A, inset) due to a shift in the proportion of Car$^+$ species, Car$_A^+$ (982 nm) and Car$_B^+$ (1029 nm) formed (2). A difference spectrum of the initial spectrum and a spectrum recorded 30 min later for each temperature of illumination is shown in Figure S4B. This double difference spectrum shows the spectrum of the Chl$^+$ species that are formed during the 15 min illumination which decay rapidly during dark incubation at these higher temperatures. In general, the absorption peaks that are less stable at 20 K are lower intensity at higher temperature. At higher temperature, activated electron transfer processes may occur. These types of electron-transfer events may be coupled to vibrational motion of the donor, acceptor or the protein. At higher temperature, the cation radical can move among the Chl and access more locations, some of which are close to Q$_A$ and result in recombination. At the warmest temperature, the Chl$^+$ absorbance maximum is $\sim$ 810 nm, which is also the wavelength most stable Chl cation radical at 20 K.

The increase in temperature is expected to result in broadening of each absorbance peak and a shift of the absorbance maximum to a longer wavelength due to a shift of the Boltzmann distribution of vibrational states to higher energy in the ground electronic state. We do observe changes in the Chl$^+$ region of the spectrum for a sample illuminated at warmer temperatures; however, because the spectrum is composed of multiple components, it is difficult to assign the absorption changes to each component. In general, the absorbance of the Chl$^+$ region at warmer illumination temperatures is lower in intensity compared to 20 K. But, as shown in Figure S4A, the change in intensity is not the same for each component.

At an illumination temperature of 20 K, the $\sim$ 810 nm and 825 nm species were the most stable and the 750 nm 793 nm and 814 nm species were less stable. In general, the 750 nm 793 nm and 814 nm peaks are lower in intensity at warmer illumination temperatures. As shown above and here for the 20 K illumination experiment, three distinct absorption peaks ($\lambda_{\text{max}}$ 750, 795 and 814 nm) decay over a period of 30 min. Each feature is less intense in the initial spectrum measured at 85 K (Figure S4, red) and are observed in the decay spectrum (Figure S4B, red). However, at illumination temperatures above 120 K, the Chl$^+$ absorbance maximum shifts to 810 nm (160 K). For the illumination temperatures of 120 K, 140 K and 160 K, the absorbance maximum of the Chl$^+$ in the decay spectra (Figure S4) have an absorption maximum of 820 nm, 819 nm, and 816 nm, respectively. This blue-shift parallels the blue-shift of the initial absorption
spectra measured at the same temperatures. At warmer illumination temperatures, the ~ 810 nm peak appears to be the most stable. We conclude that the variability of the absorption maximum reflects the contributions of several overlying features originating from chlorophyll cation radicals. The yield of each species is dynamic and depends on temperature and length of illumination.
References


Supplementary Figure Legends

**Figure S1:** EPR measurements of O$_2$-evolving PS II core complexes. (A) X-band EPR spectra of radicals generated by illumination of the sample at 30 K for the indicated illumination times. Full scale of the spectra is shown in (B). EPR conditions: microwave frequency, 9.38 GHz; temperature, 30 K; modulation amplitude, 4 G; microwave power, 0.004 mW. The Chl concentration is 2.1 mg Chl/mL, pH = 6.0 and the sample contains 5 mM ferricyanide.

**Figure S2:** Near-IR spectra of Mn-depleted PS II core complexes that were Mn-depleted by incubation with ascorbate for 5 hours at 4 °C in the dark. The sample contains 5 mM ferricyanide to oxidize Cyt b$_{559}$. Chl concentration is 0.14 mg Chl/mL.

**Figure S3:** Near-IR spectra of O$_2$-evolving PS II measured at 20 K following 15 min illumination. (A) near-IR spectra of PS II illuminated at 20 K at pH 6.0, black; pH 6.5, red; pH 7.0, green; pH 7.5, blue; pH 8.0, cyan; pH 8.5 pink. (B) expanded scale over 700 - 900 nm of the spectra shown in (A). (C) near-IR spectra of PS II illuminated at 20 K at pH 8.5 for samples in which Y$_D$ is either pre-oxidized (red) or partially reduced (black). Because Cyt b$_{559}$ can be photoreduced by the procedure to make oxidized Y$_D$, we added additional ferricyanide to the sample after room temperature illumination. The Chl concentration is 0.14 mg Chl/mL and the samples were treated with 5 mM ferricyanide prior to freezing.

**Figure S4:** Near-IR light-minus-dark difference spectra of O$_2$-evolving *Synechocystis* PS II illuminated by white light for 15 min at 20 K (black), 85 K (red), 120 K (green), 140 K (blue) and 160 K (orange) (A). Intensity of Car$^+$ formation is similar to previous measurements on Mn-depleted PS II and spectra are shown in the inset. Difference spectra of the initial spectrum minus a spectrum collected 30 min later shown in (B). Spectral changes occurring in the Car$^+$ region (920 nm – 1100 nm) are shown in the inset. The Chl concentration is 0.42 mg Chl/mL. Samples were treated with 5 mM ferricyanide prior to freezing.
Figure S1

**Figure S1**

(A) EPR Signal Intensity vs. Field (Gauss) for different time intervals:
- 1000 s
- 100 s
- 5 sec
- 0.5 sec
- 50 ms
- 10 ms
- 8 ms
- 6 ms
- 4 ms
- 2 ms
- 1 ms

(B) EPR Signal Intensity vs. Field (Gauss) for a different range of time intervals:
- 1000 s
- 100 s
- 5 sec
- 0.5 sec
- 50 ms
- 10 ms
- 8 ms
- 6 ms
- 4 ms
- 2 ms
- 1 ms
Figure S2

Diagram showing Δ Absorbance vs Wavelength (nm) for two different samples labeled A and B.
Figures S3A and S3B
Figure S3C
Figure S4

Δ Absorbance (Light-minus-Dark) vs. Wavelength (nm)

A

B

ΔΔ Absorbance