DOI: 10.1007/s11099-017-0760-x PHOTOSYNTHETICA 56 (1): 275-278, 2018

BRIEF COMMUNICATION

The deep red state of photosystem II in *Cyanidioschyzon merolae*

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Abstract

We identified and characterised the deep red state (DRS), an optically-absorbing charge transfer state of PSII, which lies at lower energy than P680, in the red algae *Cyanidioschyzon merolae* by means of low temperature absorption and magnetic circular dichroism spectroscopies. The photoactive DRS has been previously studied in PSII of the higher plant *Spinacia oleracea*, and in the cyanobacterium *Thermosynechococcus vulcanus.* We found the DRS in PSII of *C. merolae* has similar spectral properties. Treatment of PSII with dithionite leads to reduction of cytochrome (cyt) b_{559} and the *PsbV*-based cyt c_{550} as well as the disassembly of the oxygen-evolving complex. Whereas the overall visible absorption spectrum of PSII was little affected, the DRS absorption in the reduced sample was no longer seen. This bleaching of the DRS is discussed in terms of a corresponding lack of a DRS feature in D1D2/cyt b_{559} reaction centre preparations of PSII.

Additional key words: optical spectra; photosynthesis.

The splitting of water in nature is catalysed by the PSII enzyme. Light absorption is followed by transfer of excitation energy to the reaction centre (RC) of PSII, which induces charge separation and the creation of the powerful oxidant $P680^{\bullet+}$. The oxidation of two molecules of water to molecular oxygen occurs through a step-wise oxidation of the oxygen-evolving complex (OEC) *via* a redox active tyrosine residue (Tyr_Z). After four oxidation events, the OEC extracts four electrons from two molecules of water, releasing molecular oxygen whilst returning the OEC to its least oxidised configuration. The catalytic cycle of PSII is described in terms of a series of intermediate (S_i) states, where the subscript i denotes the number of stored oxidising equivalents. The process is considered to be very similar in all oxygenic photosynthetic organisms.

Great strides have been made in a broad range of studies on PSII, with the emphasis often being on the OEC. The clear goal is the elucidation of the detailed mechanism of water oxidation (Shen 2015), which will enable the development of artificial water-splitting catalysts. Many recent studies have concentrated on the prokaryotic thermophile *T. vulcanus* and its close relative *T. elongatus.* The structure of *T. vulcanus* has been determined to atomic (1.9 Å) resolution (Umena *et al.* 2011, Suga *et al.* 2015).

The only eukaryotic PSII for which a crystal structure has been obtained (Adachi *et al*. 2009, Ago *et al.* 2016) is the red algae *Cyanidium caldarium.* This structure has lower (2.76 Å) resolution than that of *T. vulcanus*, yet is clear in showing the organisation of PSII to be largely conserved between the two organisms. Red algae appear to be unique in containing a protein subunit PsbQ' which has low homology to the PsbQ subunit found in higher plants (Enami *et al.* 2008, Ifuku 2015). The water exchange kinetics of the OEC in *C. merolae*, which is

Received 14 June 2017, *accepted* 21 August 2017, *published as online-first* 3 January 2018.
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Abbreviations: Chl – chlorophyll; cyt – cytochrome; DRS – deep red state; MCD – magnetic circular dichroism; OD – optical density; OEC – oxygen-evolving complex; RC – reaction centre.
Acknowledgements: E. Krausz would like to thank Govindjee for bringing the work eventually published in reference (Pettai *et al.* 2005) to his attention whilst both attended the Photosynthesis Congress in Montreal in 2004. We recognise the support of the Australian Research Council through grants DP110104565 and DP 150103137 (E. Krausz), a Strategic Priority Research Program of CAS (XDB17030100) and a CAS Key Research Project for Frontier Science (QYZDY-SSW-SMC003) of China, and MEXT/JSPS of Japan through a Grant-in-Aid for Specially Promoted Research No. 24000018 (J.-R.S.).

closely related to *Cyanidium caldarium*, have been shown to be significantly different from those of other studied organisms (Nilsson *et al.* 2014) and point to significant (but perhaps minor) differences in the mechanism of water oxidation in OEC in red algae.

The RC present in PSII core complexes derived from *S. oleracea* and *T. vulcanus* exhibits a broad, relatively weak absorption feature that extends to 730 nm at 2 K (Hughes *et al.* 2006, Morton *et al.* 2014, Morton *et al.* 2015). Low temperature illumination of this DRS of PSII, at wavelengths longer than 700 nm, leads to charge separation and secondary donor formation in a majority of PSII centres. The absorption band is attributed to an optically accessible charge transfer state of the special pair P_{D1}–P_{D2}, and provides an alternative charge separation mechanism to the Chl_{D1} based P680 process. The DRS also exhibits broad emission, peaking at 780 nm, in both *S. oleracea* and *T. vulcanus* (Morton *et al.* 2014). The OEC of PSII possesses weak absorption in the 700–900 nm spectral range and thus overlaps with absorption of the DRS. The OEC of *T. vulcanus* has been found to exhibit well structured and distinct magnetic circular dichroism (MCD) in the S_1, S_2, and S_3 states attributable to Mn(IV) spin flip $^4A \rightarrow {}^2E$ absorption in Mn_4CaO_5 catalytic cluster of the OEC (Morton *et al.* 2015, Morton *et al.* 2017).

Parallel to the initial discovery of the low temperature spectral characteristics of the DRS were experiments made on plant leaves of *Helianthus annuus* and *Phaseolus vulgaris* at room temperature, which showed that oxygenic photosynthesis could occur using light with wavelengths as long as 780 nm (Pettai *et al.* 2005). The existence of a similar low-energy, optically accessible charge transfer state has also been demonstrated in PSI (Schlodder 2014, Morton 2015). The varied phenomenologies of low energy states in photosynthesis is of significant current interest (Reimers *et al.* 2016).

PSII from *C. merolae* was prepared using methods previously described (Adachi *et al*. 2009, Ago *et al.* 2016) and measured on a laboratory-constructed spectrometer system, which has the important advantage of being able to measure simultaneous absorption and MCD spectra at cryogenic temperatures with high sensitivity and precision (Krausz 2013). Samples were prepared to possess either a high or a low optical density (OD) by mixing PSII cores of different concentration with a 45% (v/v) glycol/glycerol (1:1, v/v) glassing medium. High OD samples were prepared using ~3.3 mg(Chl *a*) ml^{-1} concentration and were measured in a 1-mm path length hydrophobic cell. Low OD samples were prepared using either ~3.3 or ~0.18 mg(Chl *a*) ml^{-1} and measured in a 0.07-mm path length or a 1-mm path length hydrophobic cell, respectively. These were loaded and handled in low light before being dark-adapted for 5 min and subsequently cooled to 4 K over a ~1 min period. Absorption and MCD spectra were recorded at 1.8 K and 40 K. Measurements were also made on dithionite-reduced samples for which 5 µl of a ~22 mg(dithionite) ml^{-1} was added to 54 µl of cryoprotectant solution immediately before the addition of PSII cores.

Low-temperature photo-illumination experiments were also performed. For these experiments, very low measurement light fluences were used, ensuring that the absorption spectra recorded before and after illumination of the sample with intense green light were not affected by the measurement light itself. Full details of the protocols used are described by Hughes *et al.* (2006). These non-actinic measurements enable precise identification of the $Pheo_{D1}$ Q_x absorption, which undergoes a strong electrochromic shift (historically called the 'C550' shift) (Butler and Okayama 1971). Upon photo-illumination a range of other shifts and bleaches associated with the reduction of the plastoquinone Q_A to its anion (Q_A^-) and the creation of a secondary donor occur (Hughes *et al.* 2010). This secondary donor may be a Chl *a*, a reduced cyt b_{559} or a carotenoid in the RC.

The absorption changes seen upon photo-illumination shown in Fig. 1 (central thin grey line) are very well resolved and parallel corresponding measurements made on PSII in *S. oleracea* and *T. elongatus* (Krausz *et al.* 2005, Hughes *et al.* 2010). The observed highly structured features enable immediate identification of the $Pheo_{D1}$ Q_x transition at 547.5 nm and the Chl_{D1} Q_y exciton at 684.2 nm by means of their distinctive electrochromic shifts (Hughes *et al.* 2008). These shifts are induced by illumination of the low temperature sample, which causes charge separation and subsequent metastable Q_A^- formation. The fine structure seen in the 600–670 nm region is due to the vibrational sideband structure built upon the Chl_{D1} shift.

Dithionite treatment of a PSII preparation is a well-established method of Q_A and cyt reduction (Cox *et al.* 2009). In addition, the dithionite treatment causes the disassembly of the $CaMn_4O_5$ cluster of the OEC. The dithionite reduction of Q_A in *C. merolae* PSII was confirmed by absorption changes observed in the Q_y region, which mimic those induced by photo-illumination (Fig. 1). The reduced forms of cyt b_{559} and cyt c_{550} absorb in the 550 nm region where pheophytin Q_x excitations of $Pheo_{D1}$ and $Pheo_{D2}$ also occur. In the dithionite-treated sample, the $Pheo_{D1}$ Q_x transition was in its electrochromically-shifted position, creating a rather congested spectrum in the 550 nm region. In the corresponding MCD spectrum, both reduced cyts give rise to MCD A-terms. An A-term MCD feature has the shape of the derivative of the absorption profile of the band inducing the MCD (Piepho and Schatz 1983). The pair of A-terms derived from reduced cyt absorptions is clearly seen in Fig. 1 in this region. These enable an assignment of cyt b_{559} to 554.5 nm and cyt c_{550} to 545.7 nm, providing better precision than previous room temperature measurements (Krupnik *et al.* 2013).

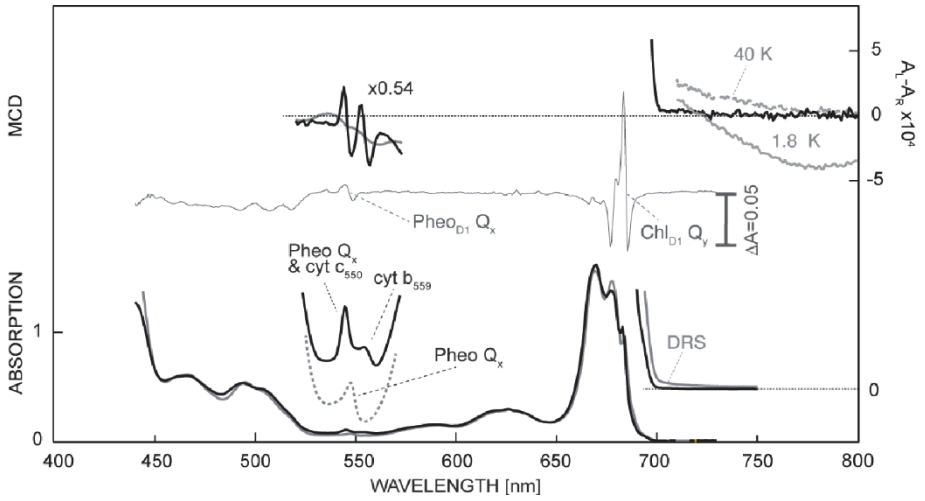

Fig. 1. Absorption (lower, left hand scale) and MCD (upper, right hand scale) of PSII cores derived from *C. merolae*. Black traces show dithionite-treated samples, whilst grey traces are samples not treated with dithionite. The absorption spectrum measured in the region between 440–730 nm was measured using a low OD sample, whilst the two detailed spectra between 520–590 nm and 690–750 nm were measured in a high OD sample (*see* text for definitions of the low and high OD). MCD were taken in an applied field of 5 T. MCD of the cyt region around 550 nm was performed on a low OD sample. MCD of the DRS was measured with a high OD sample. The central thin grey trace shows the change in absorption (scale indicated) upon illumination with green light with fluence of ~1 mW cm^{-2} for a period of 5 min at 1.8 K. All spectra shown are at 1.8 K except for the non-dithionite-treated DRS MCD for which spectra at both 1.8 K and 40 K were measured as indicated.

Absorption and MCD spectra of the nonreduced sample in the post 700 nm region revealed the broad, weak absorption characteristic of the DRS as seen in *S. oleracea* and *T. vulcanus*. The DRS in *C. merolae* has ε ~4,000 M^{-1} cm^{-1} at 702 nm, while the MCD has Δε of ~13 M^{-1} cm^{-1} at 5 T which is similar to the values of other organisms previously studied (Krausz *et al.* 2005, Morton *et al.* 2015), although the tail seems to extend further to the red in *C. merolae*. Thus, the DRS has similar absorption and MCD properties in higher-plants, cyanobacteria, and algae. Its function in PSII, if any, is not yet known. Significantly, treatment of the sample with dithionite removed the characteristic absorption of the DRS in the 700–730 nm region, as shown in Fig. 1.

The MCD of the DRS in the untreated sample shown in Fig. 1 was taken at 1.8 K and 40 K. Between 20 K and 40 K, the MCD in the near infrared region is dominantly temperature independent, as expected for the DRS. By contrast, MCD spectra of this region measured at 1.8 K to 20 K in *C. merolae* exhibited a strong negatively signed and markedly temperature-dependent MCD. This temperature-dependent signal was absent in the dithionite-treated sample. The MCD of the dithionite-treated sample shown in Fig. 1 was taken at 1.8 K. We attribute the temperature-dependent signal seen in the 700–850 nm region to most likely a cyt-based MCD, which is not present in *S. oleracea* or *T. vulcanus*. This temperature-dependent MCD is too intense to be associated with d–d transitions in the Mn_4CaO_5 cluster (Morton *et al.* 2014). Spectroscopic measurements of PsbV isolated from *C. merolae* and of PSII in which the Mn_4CaO_5 cluster has been removed would help clarify this matter and are currently being pursued. The opportunity also exists to search for ~780 nm fluorescence from the *C. merolae* DRS. A broad emission from the DRS has been identified in both *S. oleracea* and *T. vulcanus* (Morton *et al.* 2014).

The disappearance of the DRS state of *C. merolae* upon dithionite reduction may be attributed to its charge transfer characteristic. The excitation energy of an optically-allowed charge transfer state is exquisitely sensitive to its dielectric environment. An indication of the extreme sensitivity of the DRS to changes in PSII is the drop in intensity of the DRS of 30% upon (photo-illumination induced) Q_A^- formation (Hughes *et al.* 2007). It has also been noted (Krausz *et al.* 2008) that isolated RCs (Nanba and Satoh 1987) of PSII D1D2/cyt b_{559} – which are stripped of proximal antennae CP43 and CP47, and have no Q_A or redox-active Tyr_Z – show no equivalent DRS in either their RC-6 form (which contains 6 Chl *a*) or RC-5 form (in which a ChlZ is removed). The Chl *a* Q_y absorptions of the isolated RCs are blue-shifted compared to those seen in intact PSII. Accordingly, any DRS equivalent to that seen in functional PSII should be easily visible. It is likely that the removal of PsbQ', and Q_A reduction conspire to produce a significant shift of the DRS to a higher energy, and thus into a spectral region where it is not easily seen amongst the more intense Q_y absorptions of Chl *a* in PSII core complexes. Another possibility is that dithionite reduction leads to sufficient disruption to the PSII protein so that the electronic overlap of the special pair P_{D1}–P_{D2} is diminished to a point where the optical intensity of the charge transfer state involving these two Chl *a* molecules becomes inhibited.

We have shown that the DRS, *i.e*. the lowest energy state of the RC of PSII in the (red algae) *C. merolae*, has properties entirely analogous to those previously seen for the DRS in (plant) *S. oleracea* and (cyanobacterium) *T. vulcanus*. The DRS was confirmed to be a universal characteristic of functional (*i.e.* oxygen-evolving) PSII. Dithionite treatment of PSII in *C. merolae* led to a dramatic reduction in the intensity of DRS absorption in the 700–730 nm range, drawing comparisons to the lack of DRS absorption in (nonfunctional) D1D2/cyt b_{559} PSII preparations. The utility of precision low-temperature absorption, absorption difference, and MCD spectroscopies is highlighted as being able to make assignments in spectrally congested regions. The Q_x absorptions of pheophytins become distinguishable from overlapping cyt features near 550 nm and the DRS from Q_y, Mn_4CaO_5 based and cyt absorptions in the 700–900 nm region.

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