Novel characteristics of persistent spectral hole-burning and hole-filling in Photosystem II core complexes

Joseph L. Hughes, Elmars Krausz*

Research School of Chemistry, The Australian National University, Canberra, ACT 0200, Australia

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Abstract

We report spectral hole-burning as well as spontaneous and photo-induced hole-filling in Photosystem II (PSII) core complexes. A comparison is made between measurements on initially dark-adapted PSII cores in which the plastoquinone Q_A is either neutral or photo-reduced to Q_A^- by illumination at 1.7 K, with samples where Q_A is reduced by either 260 K illumination or chemical treatment. The latter preparations have the advantage of exhibiting minimal change in Q_A^- with either time or illumination at 1.7 K. This stability allowed us to investigate the association of rapid hole-filling reported for PSII core complexes with the spontaneous re-oxidation of Q_A^- , which occurs in dark-adapted samples after 1.7 K illumination. We find that spontaneous hole-filling also occurs in PSII with stable Q_A^- configurations. In either class of sample, spontaneous hole-filling occurs at significantly greater rates than for ‘normal’ two level system hole-burning processes in chlorophyll-protein antenna complexes. It is suggested that the rapid spontaneous hole-filling is associated with protein relaxation within the energy landscape of the PSII core proteins. This landscape may also be involved in influencing other processes such as low-temperature Q_A reduction and re-oxidation. Shallow holes burnt in Q_A samples exhibit a narrow ($\sim 2\text{ cm}^{-1}$) photoproduct on the low energy side of the hole. Subsequent illumination of holes burnt at 690 nm with 705 nm light induces strong hole-filling. These results are discussed with respect to charge and excitation energy transfer processes in PSII cores.

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1. Introduction

Oxygenic photosynthesis occurs via the unique ability of Photosystem II (PSII) to oxidise water [1]. The ability of this trans-membrane metallo-enzyme to extract electrons from water is essential in enabling most life on earth, yet critical aspects of the photosynthetic reaction centre in PSII remain elusive. Spectroscopic work has benefited recently from the availability of medium resolution crystal structures of cyanobacterial PSII [2–6]. The minimal photosynthetic assembly capable of oxidising water is a PSII core. Detergent-solubilised PSII cores, when prepared appropriately [7], can exhibit the same oxygenic activity as the parent membrane-bound PSII material. The principle components of PSII core complexes are the D1, D2 and cytochrome b_{559} (Cyt b_{559}) reaction centre protein subunits and the tightly bound proximal light-harvesting proteins

CP43 and CP47. There are 3 chlorophyll a molecules (Chl a) and one pheophytin a (Pheo a) in each of D1 and D2. In plant PSII core preparations the CP43 and CP47 contain 12 and 14 Chl a , respectively [7]. This is slightly fewer than the 13 and 16 Chl a contained in CP43 and CP47 as identified in the crystal structure of cyanobacterial PSII [2–6]. This discrepancy is most likely due to the loss of less well bound Chl a in the plant PSII core preparation procedure.

We have reported reproducible and well-structured low-temperature absorption [7–11], emission [10–12], circular dichroism [7–10] and magnetic circular dichroism (MCD) spectra [7,10,13], as well as persistent spectral hole-burning (PSBH) [10,14–17] and fluorescence line narrowing spectra [12] of PSII cores isolated from spinach. PSBH experiments [14–17] have proved invaluable and were instrumental in the discovery [15,18] that charge separation could be induced in PSII with radiation of far longer wavelengths than anticipated. Specifically, excitation of the supposedly ‘trap’ CP47 Chl a at wavelengths as long as 700 nm leads to

*Corresponding author. Tel.: +61 2 6125 3577; fax: +61 2 6125 0750.
E-mail address: krausz@rsc.anu.edu.au (E. Krausz).

efficient charge separation in PSII and complete photoconversion of the sample. Additionally, PSBH experiments [17] clearly establish that inhomogeneously broadened absorption of the CP47 ‘trap’ does not extend to wavelengths longer than 704 nm.

Charge separation in PSII core complexes is followed by photoconversion with the formation of a metastable Q_A^- (anion). The extent of Q_A^- formation was monitored by electrochromic shifts in Pheo *a* spectral features. Photoconversion of 80% of a PSII core sample was achieved [19] with 708 nm excitation and 30% conversion using 728 nm light. These conversions were achieved at 1.7 K, utilising a low absorbed power ($<2\text{ mW/cm}^2$) and did not reach saturation. Efficiencies of photoconversion decreased significantly with excitation wavelengths below 700 nm [19]. A homogeneously broadened absorption extending to $\sim 730\text{ nm}$ was identified and assigned as either a weak exciton band of PSII or an optically accessible, redox-tunable charge-transfer transition between the ‘special pair’ of reaction centre Chl *a* molecules in PSII [19].

We have recently reported [17] PSBH experiments performed in the 690–730 nm range, using highly active PSII cores poised in the S1 state of the PSII Kok cycle (see, for example, [1] and references therein for discussion of the Kok cycle). There was no measurable PSBH with excitation at wavelengths longer than 704 nm. This result is consistent with *inhomogeneous* broadening of CP47 ‘trap’ absorption and a dominantly *homogeneous* broadening of this low energy state. This state is likely to be a dominantly monomeric excitation of a single Chl *a*. Changes in the overall intensity of absorption in the 704–730 nm region were observed [11,17]. These changes scaled with fractional photoconversion of the sample to the metastable Q_A^- configuration. The changes seen were *independent* of the excitation wavelength used and are not attributable to PSBH processes.

Spontaneous hole-filling for holes burnt at 690 nm in PSII cores that were pre-illuminated with green light tracked the spontaneous re-oxidation of Q_A^- [17]. There was a $\sim 20\%$ ‘roll-back’ of Q_A^- that occurred over a period of 20 min. By contrast, holes burnt at 700 nm in the LHC1-730 trap states of a plant PSI sample (our unpublished results), at 670–685 nm in the CP43 proximal antenna protein (unpublished results) or at 670 nm in Chl *a* doped in polystyrene [20] show virtually no hole-filling over this time-span. The correlation of Q_A^- ‘roll-back’ with spontaneous hole-filling suggested that hole-filling in the proximal antennae was associated with charge recombination processes in the reaction centre. This idea was consistent with our proposal [15–17] that the high efficiency PSBH process in PSII cores was induced by charge separation in PSII following a slow transfer of excitation from CP43 and CP47 proximal antennae to the reaction centre. In this paper we extend our investigation of hole-filling to PSII samples in which Q_A^- is stable. We also test our prediction that narrowband excitation in the 704–730 nm region, which gives rise to charge separation in a majority of PSII

cores without inducing PSBH, will induce significant changes in spectral holes burnt at considerably shorter wavelengths.

2. Methods

The preparation and biochemical characterisation of high oxygen-evolving activity PSII core complexes from spinach have been reported [7]. High quality optical samples were prepared using 40% 1:1 ethylene glycol/glycerol as cryoprotectant in a 0.2 mm quartz cell with the protein concentration adjusted to provide a peak OD in the Q_y region of ~ 1.6 – 2.0 . Samples were dark-adapted at 290 K for 5 min to poise PSII in the S1(Q_A) configuration. Illumination of the dark-adapted sample at 260 K for 3 s with green light (see below) leads to $>95\%$ conversion of the sample to the S2(Q_A^-) configuration. This configuration is stable at low temperatures and displays a characteristic EPR ‘multiline’ signature [7]. Further illumination of an S2(Q_A^-) sample at low temperature leads to little ($<5\%$) additional Q_A reduction. Chemically reduced Q_A^- samples were prepared by adding freshly prepared sodium dithionite to a concentration of 14 mg/mL at pH 6.5 in an argon atmosphere. These reduced samples exhibited high yields of the EPR ‘split signal’ associated with the formation of a Pheo *a* anion [21] upon 200 K illumination.

The generation of samples with very low optical scatter was facilitated by rapid cooling to 4 K over $\sim 40\text{ s}$. Spectral measurements were made in transmission on a facility previously described [22]. The linewidth of the Spectra-physics 375 dye laser used ($\sim 1\text{ cm}^{-1}$) matched that of the scanning spectrometer. The dye laser was operated with DCM dye dissolved in 1:1 propylene carbonate/ethylene glycol and a 3-plate birefringent filter for wavelength selection and was pumped by a Spectra Physics model 171 Ar^+ laser. Care was taken to eliminate any background dye fluorescence or pump laser light from the exciting dye laser beam [19]. For green illumination, light from a 150 W halogen lamp was passed through a 10 cm water bath and green filter stack and focused at the sample. There was 5 mW/cm^2 peaking at 540 nm with a FWHM of $\sim 50\text{ nm}$. The green illumination was used for S1(Q_A) to S1(Q_A^-) conversion at 1.7 K and for hole-filling experiments.

3. Results and discussion

Absorption spectra of PSII cores are presented in Fig. 1 for the Q_y region, along with data for the far weaker Q_x transition near 545 nm of the two Pheo *a* molecules in PSII. In the three spectra in which the plastoquinone is reduced (present as Q_A^-), the Pheo *a* Q_x absorptions are almost quantitatively reproduced and peak near 546 nm. For the Q_A^- configuration, the peak is somewhat broader and shifted by $\sim 2\text{ nm}$ ($\sim 70\text{ cm}^{-1}$) to the blue. Conversion of samples from Q_A to Q_A^- configurations can be quantitatively traced by monitoring this spectral shift, which is due to electrochromism of the Pheo *a* bound to the D1 protein

Fig. 1. Absorption spectra at 1.7 K of spinach PSII cores prepared via specific protocols (see text) with each scaled to unity absorbance near 668 nm and offset for clarity. The top trace is of a sample in which the plastoquinone Q_A had been reduced by dithionite treatment (see text). The lower traces are spectra of fully active PSII cores prepared to poise Q_A in either a reduced or oxidised form as indicated (see text).

(Pheo_{D1}). There is no clear evidence for significant electrochromism associated with Pheo_{D2}, which is 0.23 nm from Q_A while Pheo_{D1} is much closer at 0.13 nm.

In the dithionite reduced sample, an additional peak appears at 556 nm which can be attributed to a quantitative reduction of the Cyt_b₅₅₉ heme in PSII to the diamagnetic Fe(II) form. Cyt_b₅₅₉ is fully oxidised in active cores prepared from spinach [7]. MCD measurements (data not shown) establish that <1% of the chromophore remained in the Fe(III) paramagnetic form. Spectra of all core samples in the Q_y region are similar and all exhibit a prominent feature near 683.5 nm. Electrochromic shifts become evident in the difference spectra obtained after illumination of the sample [8,10,19] and all spectra exhibit identical CP47 ‘trap’ absorption in the 690–700 nm region.

Spectral holes burnt near 690 nm exhibit a homogeneous linewidth of 1–2 GHz and have a distinctive $\sim T^2$ temperature dependence [16]. We have reported that PSHB is remarkably efficient [15,16], especially for burning when PSII is in the Q_A configuration. In this case, hole-burning and Q_A^- formation are induced at rates with initial quantum efficiencies of ~ 0.01 and ~ 0.1 – 1 , respectively [8,15,19]. The higher efficiencies in the Q_A state were attributed to the charge-separation mechanism of hole-burning. In this work, we use relatively broad ($\sim 1 \text{ cm}^{-1}$) laser excitation. This facilitates the monitoring of electrochromic shifts (the status of Q_A), phonon wings and photoproducts along with spectral holes and hole-filling via a scanning transmission spectrometer. This system has shot-noise limited sensitivity ($\Delta A \sim 10^{-4}$) and by defocusing the read-out beam, actinic affects associated with hole read-out are reduced to a negligible level.

We previously reported [17] that the decrease in depth of a hole burnt at 690 nm in a $S1(Q_A^-)$ sample quantitatively

Fig. 2. The top three panels present spontaneous spectral hole filling data at 1.7 K for 690 nm holes burnt into the PSII configurations indicated (see text). The hole depth is $\Delta A/A_0$, where A_0 = pre-burn absorption. The hollow data points are for deep holes (34–39%) burnt with 2.4 J/cm^2 fluence and the solid points for shallower (9–14%) holes burnt with 8 mJ/cm^2 fluence. The first illumination was sufficient to induce complete photoconversion of an $S1(Q_A)$ sample and the latter illumination converted $\sim 37\%$. The bottom panel monitors Q_A^- from electrochromism in the Q_x transition of Pheo_{D1} (see text). The hollow data points follow a 1.5 J/cm^2 illumination with green light and the solid curve a 5 mJ/cm^2 illumination. The former illumination led to complete photoconversion of the sample and the latter to 43%.

tracked spontaneous re-oxidation or ‘roll-back’ of Q_A^- to Q_A . Fig. 2 provides hole-filling data for holes burnt in PSII in the $S2(Q_A^-)$ configuration and shows that spontaneous hole-filling occurs at similar rates and to a comparable extent to Q_A^- ‘roll-back’. The stability of Q_A^- in this sample was confirmed by monitoring Q_x Pheo_{D1} electrochromism. Hole-filling is clearly independent of the process of spontaneous Q_A^- re-oxidation which we call ‘roll-back’. In dithionite reduced cores, where Q_A^- is also stable, spontaneous hole-filling (data not shown) was similar to that in the $S2(Q_A^-)$ sample. We also see that when a hole is burnt when PSII is in the Q_A state (Fig. 2), where hole-burning is most efficient, the spontaneous hole-filling process is qualitatively the same.

Fig. 2 also shows that spontaneous hole-filling kinetics are dependent on hole-depth. Shallower holes exhibit a larger fraction of a faster component, in both the $S1(Q_A^-)$ and $S2(Q_A^-)$ configurations. A comparable phenomenology is seen in Q_A^- ‘roll-back’ kinetics. A sample fully converted to the Q_A^- configuration exhibits a smaller fraction of the faster kinetic component than a sample in which $\sim 40\%$ of Q_A is converted (Fig. 2, bottom panel). In order to achieve the $\sim 40\%$ conversion, the sample had absorbed approximately

the same number of photons as used for shallow hole-burning. Likewise, the 100% Q_A^- conversion utilised approximately the same number of absorbed photons as the deep hole-burning shown in Fig. 2. Q_A^- ‘roll-back’ is not necessary for rapid spontaneous hole-filling, in contrast to our previous association [17], and yet both processes exhibit the same phenomenology. We suggest that the relaxation of both the hole-burned and charge separated Q_A^- states appear to explore the dispersion in the topology of the energy landscape of the protein in comparable ways. When a protein at physiological temperatures is rapidly frozen, the range of conformations normally accessible to the protein (its ‘landscape’) is sampled. Each characteristic subset of now *static* conformations may show a particular photoconversion, hole-burning or other characteristics.

The dependence of the spontaneous hole-filling rate on the hole depth for holes burned in the $S_1 \leftarrow S_0$ transition of organic molecules in glassy hosts has been reported [23]. In systems where there is little or no spectral diffusion, the spontaneous hole-filling has been associated with a correlation of the hole and anti-hole (photoproduct) [23,24]. Since there is a large dispersion of burning rates in such systems, shallow holes are burnt more easily and due the correlation of hole and product, this leads to a faster filling of the shallower holes. To our knowledge there has been no report of such hole-filling phenomena in Chl-protein systems. We note that the minimal spectral diffusion which is observed (<20% of the homogeneous holewidth) occurs within ~ 2 min for holes burnt in PSII core complexes in the $S_1Q_A^-$ configuration [15]. The dispersion in hole-burning and spontaneous hole-filling rates may be a property of the topology of the energy landscape of the protein.

In Fig. 3 we present spectra of holes burnt at 690 nm in PSII cores poised in four different redox configurations. Holes were burnt with similar fluences. When PSII is poised in the $S_1(Q_A)$ configuration hole-burning is most efficient and the shallow holes exhibit a distinctive very narrow photoproduct to lower energies. A less pronounced narrow photoproduct is evident in the sample in which 50% of the PSII centres had been converted to the Q_A^- state. This narrow, red-shifted photoproduct is also evident for shallow hole-burning at shorter wavelengths in the S_1Q_A configuration (data not shown). We previously identified [15] two distinct photoproduct distributions for hole-burning in PSII core complexes. The broad photoproduct is distributed across the entire inhomogeneous distribution of hole-burned pigments, and dominates for hole-burning in the Q_A^- configuration. The narrow photoproduct is distributed from the resonant hole by ~ 2 – 5 cm^{-1} [15], and is identified in the present work as originating from Stark shifts on the laser-selected pigments due to the presence of Q_A^- and/or the secondary donor. The broad photoproduct distribution is not evident over the spectral range shown in Fig. 3.

We performed photo-induced hole-filling experiments on samples that were let adapt at ~ 2 K in the dark for ~ 2 – 3 h

Fig. 3. Spectral holes burnt near 690 nm in PSII samples and scaled to the same depth. The depth of each hole is indicated (the hole depth is $\Delta A/A_0$, where A_0 = pre-burn absorption). The top spectrum was burnt with 25 mJ/cm^2 at 690.3 nm and the others with 8 mJ/cm^2 at 690.1 nm. The reduced and $S_2(Q_A^-)$ samples were generated as described in the text. The fully converted $S_1(Q_A^-)$ sample was created by 1.5 J/cm^2 green light illumination and the hole was burnt and read-out within ~ 3 min. The 50% converted $S_1(Q_A^-)$ sample was created by 1.5 J/cm^2 green illumination followed by a ~ 3 h dark period in which the sample relaxed (see Fig. 2). The hole-burning in $S_1(Q_A)$ led to $\sim 37\%$ Q_A^- formation.

after the initial hole-burning. After such dark adaptation, the remaining holes are reasonably stable over a ~ 10 – 20 min time period and exhibit negligible spontaneous hole-filling during the time needed for illumination. The full range of PSII redox configurations utilised (Fig. 1) exhibited comparable photo-induced hole-filling with broadband green light. Illumination corresponding to ~ 2000 absorbed photons/PSII over a 5 min period was found to fill shallow holes ($\sim 5\%$) entirely. Illumination at energies below that of a persistent spectral hole and outside the range of the chromophore photoproduct distribution would not be expected to lead to hole-filling for holes burnt via the regular two level system mechanism [25,26]. Our association of the PSHB process in PSII with charge separation, which occurs *following* excitation energy

Fig. 4. The lower trace shows a spectral hole burnt near 690 nm in an $S1(Q_A^-)$ sample by a fluence of 8 mJ/cm^2 . The middle trace shows the hole after $\sim 2 \text{ h}$ in the dark at 1.7 K . The top trace shows the hole following illumination of the relaxed hole (middle trace) with 27 J/cm^2 at 705 nm . The top and middle traces have been offset for clarity, and the hole depths are indicated.

transfer of the hole-burning pigment to the reaction centre, lead us to predict that direct excitation of the reaction centre via low-energy illumination may influence holes at far higher energies. Fig. 4 provides a clear demonstration that illumination of a 690 nm hole-burned $S1(Q_A^-)$ sample with light at 705 nm leads to strong hole-filling. With an absorbed fluence corresponding to ~ 1000 photons/PSII, $\sim 65\%$ of the hole was filled and in particular the narrow red photoproduct vanished. Holes burnt in the other PSII redox configurations (Fig. 1) showed minimal hole-filling under the same illumination.

For holes burnt in an initially $S1(Q_A^-)$ sample, hole-filling induced via illumination at 705 nm can be rationalised by noting that excitation at 705 nm can give rise to photo-conversion of at least $\sim 80\%$ of centres [19]. The lack of selectivity in this process is evidenced by the absence of PSBH. The phenomenology seen is consistent with the narrow, red-shifted photoproduct being due to CP47 pigments shifted to lower energy by the electric field associated with the subsequent formation of Q_A^- and/or its partnering secondary electron donor.

An electrochromic shift of $2\text{--}5 \text{ cm}^{-1}$ is compatible with calculations based on the interaction of the $\Delta\mu$ of the 'linker' CP47 Chl a 's with the summation of electric fields arising from Q_A^- and the secondary donor. Either of the D1/D2 accessory Chl z and the D1/D2 β -carotenes were considered as potential donors. The 'linker' pigments are those closest to the reaction centre [27,28] and for CP47 are

Chl24 and Chl21 in the notation of Loll et al. [3]. The relevant distances and angles required for the calculation were determined from the structural data of Loll et al. [3] (PDB entry 2AXT), and we used $\Delta\mu = 1$ Debye and a protein dielectric constant of 1. This results in a calculated red-shift of $\sim 20\text{--}45 \text{ cm}^{-1}$ for Chl24 and a blue-shift of $\sim 0\text{--}15 \text{ cm}^{-1}$ for Chl21.

The magnitude of the electrochromic shift of the photoproduct is dependent on which secondary donor is formed, as although any donor will have unit positive charge, this charge will be at a different distance and relative orientation from the pigment under consideration. The sign of the shift that we observe is to lower energy and is thus consistent with the CP47 'trap' being Chl24. The magnitude of the calculated shift will be decreased by a more appropriate estimate for the protein dielectric constant. A value of $\epsilon \sim 10$ would produce a red-shift of Chl24 by $\sim 2\text{--}5 \text{ cm}^{-1}$.

Illumination with 705 nm light following shallow hole-burning at 690 nm leads to Q_A^- formation in a majority of PSII. Thus, the initial selectivity offered by laser excitation at 690 nm , i.e. photoconverting those PSII centres that were laser-selected from within the inhomogeneously broadened CP47 distribution is lost and the spectral hole disappears. A 2 cm^{-1} shift of the $\sim 250 \text{ cm}^{-1}$ FWHM inhomogeneous distribution of CP47 is too small to be identified in the presence of other photophysically induced spectral changes in this range upon Q_A^- formation.

The hole that remains after the 705 nm illumination (Fig. 4) does not exhibit a narrow photoproduct. This hole can be attributed to hole-burning in the Q_A^- configuration. Q_A^- can be created *non-selectively* during the laser illumination that is used for 690 nm hole-burning. Even by burning a shallow hole at 690 nm (Figs. 3 and 4), $\sim 40\%$ of the sample is converted to the Q_A^- configuration. We have previously noted [19] that in order to account for the high yield of photoconversion observed with illumination between 690 and 700 nm , it was necessary to invoke a homogeneously broadened excitation of PSII underlying the CP47 absorption that can lead to efficient charge separation.

4. Conclusions

We have shown that spontaneous hole-filling in PSII cores remains rapid for hole-burning in redox configurations in which spontaneous re-oxidation of Q_A^- is inhibited. We suggest that the protein energy landscape may be the determining factor in hole-filling and not Q_A^- 'roll-back'. PSBH of PSII poised in the Q_A^- configuration is particularly efficient and exhibits a narrow red-shifted photoproduct. The photoproduct is provisionally attributed to an electrochromic shift of the subset of CP47 pigments selectively excited by the laser. The hole is efficiently filled by illumination at 705 nm . This process can be understood by noting that 705 nm illumination leads to charge separation in a majority of centres.

We plan to pursue the unusual aspects of PSHB presented here in more detail. An analysis of the narrow red photoproduct may provide a novel and effective way of tracking electron transfer processes in PSII.

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