Highly Efficient Spectral Hole-Burning in Oxygen-Evolving Photosystem II Preparations†

Joseph L. Hughes,‡ Barry J. Prince,§ Elmars Krausz,*‡ Paul J. Smith,§ Ron J. Pace,§ and Hans Riesen⊥

Research School of Chemistry, The Australian National University, Canberra ACT 0200, Australia, Faculties Chemistry, The Australian National University, Canberra ACT 0200, Australia, and School of Physical, Environmental and Mathematical Sciences, University College, The University of New South Wales, ADFA, Canberra ACT 2600, Australia

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We present the first report of highly efficient persistent spectral hole-burning in active (oxygen-evolving) Photosystem II (PSII) preparations. Samples are poised in the S1 state of the Kok cycle, with the primary quinone (QA) either neutral or photoreduced to QA− via a low-temperature pre-illumination. Remarkably efficient hole-burning is observed within the chlorophyll Q(0,0) absorption envelope in the wavelength range of 676–695 nm. The hole-burning action spectrum of a sample poised in the S1(QA) state is dominated by a narrow feature (~40 cm⁻¹) at 684 nm, where hole depths of 30% are attainable. The photoproduct for spectral holes burnt in this region is distributed across the ~50 cm⁻¹ absorption feature centered at 683.5 nm, independent of the excitation wavelength within this band. Saturated hole-burning experiments indicate weak electron–phonon coupling near 684 nm but stronger coupling for holes burnt near 690 nm. Selective excitation near 690 nm of samples in the S1(QA) state also results in efficient QA− formation. Negligible hole-burning activity is observed at higher energies (~676 nm). Holewidths extrapolated to zero fluence and temperature are 2.0 ± 0.5 GHz near 685 nm for PSII samples in the S1(QA) state. Holewidths are twice as large and hole-burning quantum efficiencies are up to an order of magnitude greater (approaching 1%) for samples in the S1(QB) state. We ascribe hole-burning near 684 nm to slow (~40–210 ps) excitation transfer from a CP43 chlorophyll to the PSII reaction center, and we ascribe hole-burning at ~690 nm to excitation transfer from a chlorophyll in CP47. The unusually high hole-burning efficiency that we observe is attributed to a mechanism that involves charge separation in the reaction center that follows excitation transfer from these “slow transfer” states in CP43 and CP47. A key result of this work is the observation that selective excitation in the range 685–695 nm leads to efficient charge separation, as indicated by QA− formation. This indicates the presence of (a relatively weak) P680 absorption in a native PSII, extending to low energy and underlying the CP47 chlorophyll trap absorption.

1. Introduction

1.1. Photosystem II and P680. Photosystem II (PSII) is a complex multiprotein transmembrane assembly found in all O₂-evolving photosynthetic organisms. The entire PSII assembly consists of a core complex, and a system of light-harvesting antenna proteins. A fully functioning PSII core complex consists of pigment-containing D1, D2, CP43, and CP47 proteins, and an integral cytochrome b₅₅₉ (cyt b₅₅₉) protein. The manganese catalytic site at which water oxidation occurs is bound to D1. Other smaller pigment-free peptides are involved in the stabilization of active PSII.1 The D1/D2 heterodimer is analogous to the L and M proteins in the bacterial reaction center2 and contains the redox-active pigments (Figure 1).

The availability of 3.4–3.8 Å resolution X-ray crystal structures3–5 of membrane-bound PSII core complexes of thermophilic cyanobacteria has significantly aided spectroscopic studies of PSII. These relatively low-resolution X-ray structural data have confirmed the general arrangement of pigments and redox centers within a cyanobacterial PSII core. There is a significant homology of the major cyanobacterial and plant PSII core proteins,2 as well as a strong similarity of cyanobacterial and plant PSII functions. This allows us to assume an analogous PSII organization in plants and cyanobacteria.

Figure 1. Arrangement of PSII reaction center chromophores taken from the crystal structure reported by Zouni et al. The four central chlorophyll molecules are labeled Pₐ, Pₐ, ChlD₁, and ChlD₂. The peripheral chlorophyll molecules are labeled ChlZD₁ and ChlZD₂, whereas the D1/D2 phytopheophytin molecules are labeled PhcD₁ and PhcD₂, respectively. The quinone electron acceptor molecule (QA) and the non-heme Fe are indicated, along with the heme group of the cyt b₅₅₉ subunit, the redox active tyrosine protein residues Tyr₁ and Tyr₁, and the Mn-cluster.
The D1 and D2 protein subunits are each comprised of five transmembrane helices and, together, bind all the pigments of the reaction center. The D1 and D2 proteins each bind three chlorophyll $a$ molecules (chl $a$) and one pheophytin $a$ (pheo $a$). One chl $a$ from each of the D1 and D2 proteins is bound to the periphery of the D1 and D2 proteins, and they are known as peripheral chl $a$, or ChlZ, leaving four central chl $a$ and two pheo $a$. The plastoquinone electron acceptors, $Q_A$ and $Q_B$, are bound to the D2 and D1 proteins, respectively. A non-heme Fe ion is located between these plastoquinones. Situated between the Mn-cluster and the four central chl $a$ pigments are two redox-active tyrosine protein residues, $D1-161 (Y_Z)$ and $D2-160 (Y_D)$, as shown in Figure 1. There are two $\beta$-carotenes in the reaction center, and crystallographic evidence indicates that both $\beta$-carotenes are within the D2 protein.\cite{6,7} The Mn-cluster is the site of catalytic water oxidization and lies at the luminal edge of the D1 protein.

The nature of the primary electron donor is currently being debated. We use the term P680 as a generic description of the photoactive assembly in PSII in either its native or isolated form. An excited state of P680 (P680*) is highly oxidizing and initiates the primary photochemistry of PSII. The degree of coupling between the four central chl $a$ (see Pd1, Pd2, ChlD1, and ChlD2 in Figure 1) and the two pheo $a$ bound to the D1 and D2 proteins (see PheoD1 and PheoD2 in Figure 1) of PSII is under continued discussion.\cite{6,7} Monomeric, dimeric, and multimeric models have been proposed for P680.\cite{6,7}

The inner antenna proteins (CP43 and CP47) are intimately associated with the D1/D2/cyt$b_{559}$ proteins. The CP43 and CP47 pigment-proteins in spinach cores are estimated to contain 12 and 14 chl $a$, respectively.\cite{1} In both CP43 and CP47, these chl $a$ molecules are arranged in two layers, which are associated with the stromal and luminal regions of the proteins.\cite{3,5} They serve as “inner” light-harvesting subunits and act as a conduit for excitation transfer from the main light-harvesting assembly to P680. The outer antenna light-harvesting assemblies of PSI are not directly involved in its redox chemistry; however, they do serve to transfer electronic excitation to P680 via CP43 and CP47.

Photoactivated electron flow only occurs through the D1 protein. Primary charge separation involves the rapid reduction of the PheoD1 by P680* to form P680$^+$PheoD1$^{-}$. Secondary electron transfer from PheoD1$^{-}$ to Q$_A$ occurs within $\sim$200 ps.\cite{6,7} P680$^+$ oxidizes the redox active tyrosine, Y$_Z$, in nanoseconds.\cite{6,7} which, in turn, oxidizes the Mn-cluster on a millisecond time scale.\cite{6,7} There are four sequential one-electron light-driven oxidative steps of the Mn-cluster in PSI, which are described by the Kok cycle.\cite{2} In this manner, the Mn-cluster is progressively advanced from the lowest oxidation state ($S_0$) to the $S_1$, $S_2$, $S_3$, and $S_4$ states. Oxygen is evolved at the catalytic Mn site in the $S_4$ state, returning the system to the $S_0$ state. The room-temperature, dark-adapted state of PSI is $S_1$.

A PSI preparation can be poised in a specific S state by appropriate illumination protocols and then trapped by rapid freezing. Detailed spectra of a range of metastable S-state species can thus be obtained. There have been numerous electron paramagnetic resonance (EPR) studies of the $S_0$–$S_1$ states at low temperatures (for recent reviews, see refs 8–11). If a PSI sample is illuminated at a temperature of $\sim$200 K, manganese oxidation is inhibited and alternative electron donors are utilized by the system.\cite{12,13} Electrochromic shifts of nearby chlorophores are associated with $Q_A^-$ radical anion formation. These are conveniently studied via low-temperature (1.7 K) illumination of a sample poised in the $S_1$ state. The $S_1(Q_A^-)$ state is formed with a high quantum efficiency ($\geq 0.1$) and is stable over a period of hours.\cite{14}

Spectral assignments of P680 remain the subject of ongoing discussion.\cite{5,7} Significant spectral congestion is inherent in the optical spectra of PSI core complexes. This is partially due to the presence of chl $a$ associated with both CP43 (12 chl $a$) and CP47 (14 chl $a$), whose absorption due to $Q_s$-state transitions is in the same spectral range as those due to the $Q_A$-state transitions of the D1/D2 chl $a$ and pheo $a$. Another contributing factor is that weaker coupling exists, particularly between the P680 and P682 pigments, compared to their analogues in the bacterial reaction centers. This results in the energy separations of coupled chromophores being less than or equal to the spectral inhomogeneity in PSI cores.

To circumvent this spectral congestion, optical experiments that seek to probe the reaction-center pigments are commonly performed on D1/D2/cyt$b_{559}$ preparations. An added advantage of these preparations is that photochemistry beyond the primary charge separation step is inhibited by the absence of secondary electron donors and acceptors. Charge recombination of P680$^+$PheoD1$^{-}$ is rapid (40–100 ns, depending on temperature) (see Groot et al.\cite{15} and references therein), allowing repeated measurement of charge-separation kinetics via transient absorption or time-resolved fluorescence, for example. In contrast, the illumination of a core complex leads to the efficient formation of relatively stable species. If measurements are made at room temperature, the sample can be continuously regenerated or replaced. However, low-temperature experiments are more difficult, as a range of photoproducts accumulate with a wide range of efficiencies. D1/D2/cyt$b_{559}$ preparations degrade with prolonged or intense illumination.\cite{16}

We have suggested that the native D1/D2/cyt$b_{559}$ assembly becomes disrupted upon the detergent reduction of a core complex into protein components.\cite{1,14,17,18} Relatively severe detergent treatment is required to remove CP43 and CP47, compared to the procedures used to make active cores. This may affect the conformation and homogeneities of the D1/D2 proteins. As a consequence, spectra of pigments in the D1/D2/cyt$b_{559}$ preparation do not fully reflect the properties of native P680 present in active PSI. In contrast, we have shown that spectral features—particularly, the prominent feature at 683.5 nm—are quantitatively retained in membrane-bound PSI and solubilized PSI core complexes.\cite{1,14} Absorption, circular dichroism (CD), magnetic circular dichroism (MCD), and electrochromism data were used to associate the prominent feature at 683.5 nm with P680.\cite{19} We have established\cite{17,20} that a CP43 absorption feature in this region cannot fully account for the 683.5 nm feature in PSI cores, unless the CP43 feature undergoes a 2-fold decrease in intensity upon isolation from the PSI core.

1.2. Charge and Excitation Transfer in the Photosystem II Reaction Center. Charge separation and subsequent photochemistry in PSI occurs by spontaneous ionization of the excited state of P680 (P680*). Numerous studies that use a range of approaches have attempted to determine rates of primary charge separation and excitation transfer processes in PSI, without a final consensus. There is much less spectral congestion and inhomogeneity in the spectra of bacterial reaction centers, compared to that of PSI. As a consequence, charge separation in the bacterial reaction center is better understood than the process in PSI.\cite{3} There is significant structural and functional similarity between the two systems and results from studies on bacterial reaction centers have often served as a model for processes in PSI.\cite{2,22} The rate for reduction of the bacteriopheo-
phytin in bacterial reaction centers, via charge separation of the primary donor near room temperature, is \( \sim (3 \text{ ps})^{-1} \).\(^{1,22}\) Excitation into any of the Q\(_y\) absorption bands in the reaction centers of purple bacteria at 10 K leads to excitation localization on the primary donor within 100 fs (see Durrant et al.\(^{23}\) and references therein).

Despite the lack of agreement regarding the rates of energy and charge transfer in PSII, some aspects have gained a general consensus. Excitation transfer among the central D1/D2 chromophores (excluding the peripheral chl a) is generally accepted to be rapid and occurs on a sub-picosecond time scale.\(^{7,22,24}\) Excitation transfer from the peripheral chl a (ChlZ) to the central four D1/D2 chromophores is thought to occur on a 10–50 ps time scale.\(^{7,22,24}\) The majority of studies discussed in the review articles referenced\(^{7,22,24}\) have reported work at or near room temperature. In native photosynthetic assemblies, the majority of P680* will form via excitation transfer from the inner antennae CP43 and CP47, following transfer from light-harvesting assemblies, rather than via the direct excitation of P680 itself.

1.3. Native Photosystem II. For the reasons outlined above (i.e., the accumulation of metastable species at low temperature), fast transient measurements on O\(_2\)-evolving PSII preparations have been made at room temperature. It has been argued that charge separation is more efficient when Q\(_y\) is oxidized (neutral).\(^{25}\) The fluorescence efficiency increases markedly (by a factor of 3) when Q\(_y\) is maintained in the oxidized state by chemical treatment.\(^{25}\) Kinetic measurements at room temperature of fluorescence and absorption changes in O\(_2\)-evolving PSII samples that contained \( \sim 80 \text{ chl} \) per core showed processes with time constants of \( \sim 80–120 \text{ ps} \) for open PSII (Q\(_y\) oxidized) and \( \sim 170–260 \text{ ps} \) for closed PSII (Q\(_y\) reduced).\(^{25}\) From these data, a model was developed\(^{26}\) in which the intrinsic primary charge separation rate was determined to be (2.7 ps)\(^{-1}\). The model explained the observed relatively slow kinetics by assuming fast (<2 ps) excitation equilibration among all the antenna chl a and the primary donor. In this trap-limited model, P680 acts as a shallow trap for excitation.

Schelvis et al.\(^{27}\) have performed time-resolved picosecond absorption measurements at room temperature on O\(_2\)-evolving PSII core preparations with \( \sim 35 \text{ chl} \) a per core. The experiments were performed on closed PSII (Q\(_y\) reduced), and they observed kinetic components with time constants of 21, 80, and 200 ps. A wavelength dependence of the intermediate component (80–200 ps) was observed, whereas the 21 ps component was observed to dominate at longer wavelengths (\( \sim 690 \text{ nm} \)). This wavelength dependence was used to argue against the possibility of fast equilibration of excitation between the entire antenna and P680. Transient experiments at 77 K on isolated CP43 and CP47\(^{28}\) have identified a \((2-3 \text{ ps})^{-1}\) rate that connects pigments bound to the stromal and luminal sides of the membrane. It was suggested that energy transfer from CP43 and CP47 to the reaction center will occur from those chl a on the stromal side of these inner antennae. Again, the \((2-3 \text{ ps})^{-1}\) kinetic component is too slow to be easily consistent with the shallow trap model for PSII cores.

1.4. D1/D2/cyt\(_{b559}\). There is ongoing discussion regarding interpretation of results obtained from studies on D1/D2/cyt\(_{b559}\) preparations. At low temperature, a primary charge separation rate of \( \sim (1-3 \text{ ps})^{-1} \) is generally accepted, whereas at room temperature, rates from \((1-3 \text{ ps})^{-1}\) up to \( \sim (20 \text{ ps})^{-1} \) are still debated.\(^{7,22,24}\) At low temperatures, the fast kinetics directly observed are multiphasic, with components ranging from \((1-3 \text{ ps})^{-1}\) up to \((20–30 \text{ ps})^{-1}\) and \((100–200 \text{ ps})^{-1}\).\(^{7,22,24}\) Fast transient experiments are prone to nonlinear effects and also sample degradation at the high powers, which are inherent in even the lowest fluence experiments.\(^{16,29–31}\)

Two-pulse photon-echo experiments\(^{32}\) were performed at 1.3 K with minimal pulse energies and were analyzed via a model to provide an intrinsic charge separation rate of \((1.5 \text{ ps})^{-1}\), although a wide range of kinetics was invoked. More direct recent experiments\(^{33}\) made at 77 K on several D1/D2/cyt\(_{b559}\) preparations have indicated a \((3.1 \text{ ps})^{-1}\) rate in the usually studied preparation and a \((0.8 \text{ ps})^{-1}\) rate in a sample where Pheo\(_{b2}\) was replaced by a chemically modified phaeophytin.

Hole-burning spectroscopy\(^{34,35}\) is a frequency-domain experiment in which the holewidth can provide information on the lifetime of the excited state(s) of the chromophores involved. A homogeneous holewidth of 1 GHz corresponds to an excited-state lifetime of 318 ps. Such experiments can be performed at very low fluence and provide a useful adjunct to fast pump–probe spectroscopies. Persistent spectral hole-burning has been performed on D1/D2/cyt\(_{b559}\), providing information on strongly fluorescent trap pigments with lifetimes of \( \sim 4 \text{ ns} \), which are present in these samples but are not involved in charge separation.\(^{15,36}\) We are aware of only one report \(^{39}\) of persistent spectral hole-burning in active PSII. These experiments were performed at a very high laser fluence, leading to shallow and broad spectral holes. We are not able to reproduce their results. The current study was undertaken to provide information on the spectral position, excited-state lifetimes, and electron–phonon coupling of longer-lived excitations within PSII cores.

2. Materials and Methods

Membrane-bound PSII-enriched material (BBY) was isolated from spinach, as previously described.\(^{3}\) PSII core complexes containing \( \sim 32 \text{ chl} \) a per core were prepared from membrane-bound PSII, also as previously described.\(^{3}\) These PSII cores have comparable O\(_2\)-evolving activity to the membrane-bound preparation. Synechocystis 6803 (syn. 6803) cyanobacterial PSII core complexes were prepared as described in Peterson Årsköld et al.\(^{14}\) These concentrated preparations (1–5 mg chl a/mL) were stored in the dark in sucrose buffers at 77 K. For spectroscopic measurements, samples were rapidly thawed then diluted in the elution buffer and mixed with an ethyleneglycol/glycerol (1:1) glassing medium to a final concentration of 40%–50%, achieving a maximal optical density of \( \sim 1 \) in the chl a Q\(_y\) spectral region. All sample handling was performed under dim green light. The prepared sample cell was left in darkness for 5 min at room temperature before rapid freezing to the data collection temperature (1.7 K). Spectral holes were measured in transmission.

For absorption spectra and broadband hole-burning measurements, a 12-mm-diameter quartz-windowed cell assembly with a path length of 150 \( \mu \text{m} \) was utilized. An Oxford Instruments Spectromag 4 cryostat was used. Glasses of high optical quality
were obtained by lowering the cell, fixed to the sample rod, into liquid helium. Cooling from 300 K to 4 K occurred over a time period of ~30 s. The liquid helium was then pumped to perform transmission experiments in bubble-free superfluid helium. Absorption features <10⁻⁴ could be reliably measured.

The spectrometer used for absorption and broadband hole-burning measurements was designed and constructed in our laboratory and has been described in detail elsewhere. The spectra presented in this report were recorded with 50-mm slit widths (0.03 nm resolution) using a Hamamatsu R669 photomultiplier tube for detection. The measurement beam was expanded to approximately the diameter of the sample cell (12 mm), to minimize actinic effects of the measurement light.

Broadband spectral holes were burnt with a Spectra-Physics model 375 dye laser that was operating with DCM dye and pumped by a Spectra-Physics model 171 Ar+ ion laser. Wavelength selection of the dye laser was achieved using a two-plate birefringent filter so that the width of spectral holes was purposely expanded to 2–3 cm⁻¹ by the line width of the laser. This matches the resolution of the spectrometer and avoids artifacts due to a sharp, unresolved hole-burning feature.

For the high-resolution spectral hole-burning measurements, the prepared sample (as above) was placed into a 1.5-mm-pathlength sapphire-windowed cell and mounted on the coldfinger of a closed-cycle refrigerator (Janis/Sumitomo SHI-4.5). The sample cooling time to ~2.5 K was ~2 h. The mounted sample was cooled to ~50 °C in <30 min and was at a temperature of ~270 K for ~10 min. A Lakeshore model 330 auto-tuning temperature controller was used to monitor a silicon diode thermometer mounted on the coldfinger of the closed-cycle refrigerator. The sample temperature was calibrated by measuring the fluorescence emission of the NaMgAl₂(oxalate)₃·9H₂O·Cr(III) crystal, as a function of temperature. The relative amplitudes of the Cr(III) R₁ and R₂-line emission are accounted for by a Boltzmann population. Hole-burning was achieved, and transmission spectra recorded, using an Hitachi model HL6738MG laser diode mounted on a Thorlabs model TCLDM9 thermoelectric mount. The current and temperature of the 690-nm laser diode were maintained and controlled by Thorlabs model LDC500 and TEC2000 current and temperature controllers, respectively. A schematic diagram of the high-resolution hole-burning apparatus is presented in Figure 2.

The frequency of single-mode laser diodes can be tuned by varying the injection current. The frequency/current tuning ratio of the diodes used in this work was on the order of ~2.5 GHz/mA. The current controller (Thorlabs model LDC500) allows for the external modulation of the injection current, for which we used a 2000 or 2500 Hz triangular waveform provided by a Stanford model SRS DS345 function generator. Mode-hop free scans of ~30–40 GHz were possible for the diodes used in the experiments reported here. A diode voltage amplitude change of ±10 mV corresponded to a current modulation of ±0.5 mA and, thus, a frequency scan of approximately 1.25 GHz. A Coherent model 240 spectroscopy analyzer, with a free spectral range of 300 MHz, was used to calibrate the wavelength scan.

Hole readout in transmission was achieved using a silicon photodiode (Thorlabs model PDA55-EC), or a Hamamatsu 3-mm model S2384 silicon avalanche photodiode. These detectors had high quantum efficiency (>70%) at 690 nm and, for this reason, were used in preference to photomultipliers. Care was taken to focus a large fraction of the transmitted laser light on the detector, to allow the highest possible sensitivity of hole-readout for a given light fluence. The transmission signal was

![Schematic diagram of the experimental diode-laser hole-burning apparatus. The diode-laser has a line width of ~20 MHz and is tuned by varying the injection current. The injection current is modulated by a triangular waveform, typically with a frequency of 2500 Hz, which is provided by a function generator. The laser light is defocused onto the sample to a beam size of ~0.6 cm². All the light transmitted through the sample is focused onto the detector, and the data are accumulated on an 11-bit digital oscilloscope and then subsequently transferred to a personal computer. (See Materials and Methods for further information.)](image)

Figure 2. Schematic diagram of the experimental diode-laser hole-burning apparatus. The diode-laser has a line width of ~20 MHz and is tuned by varying the injection current. The injection current is modulated by a triangular waveform, typically with a frequency of 2500 Hz, which is provided by a function generator. The laser light is defocused onto the sample to a beam size of ~0.6 cm². All the light transmitted through the sample is focused onto the detector, and the data are accumulated on an 11-bit digital oscilloscope and then subsequently transferred to a personal computer. (See Materials and Methods for further information.)

accumulated and averaged on either an 8-bit (y-resolution) Tektronix model TDS210 or an 11-bit (y-resolution) LeCroy model 9310 digital oscilloscope and, subsequently, on a Pentium-III-based personal computer. Under the operating conditions used, the laser line width was 20 MHz.

For hole-burning, the laser was kept at constant current and, thus, constant wavelength, and the light was attenuated by an initial neutral density (ND) filter, before being defocused at the sample to a beam size of ~0.6 cm². The reflected beam from the ND filter was monitored by a wavemeter to determine the excitation wavelength. Additional ND filters were then placed between the laser and the cryostat, to achieve the desired laser intensity for hole-burning. For hole readout, the beam was further attenuated by additional ND filters to a point where minimal additional hole-burning occurred.

3. Results

3.1. Low-Temperature Qₐ Formation. Figure 3 shows the 1.7 K absorption spectrum in the Q₁(0,0) region of a spinach PSII core complex after dark adaptation and before any illumination, with PSII poised in the S₁(Qₐ) state. The spectrum is well-structured, with a prominent sharp (~50 cm⁻¹) feature at 683.5 nm whose area corresponds to ~2 chl a₁. Illumination of the sample at low temperature leads to measurable changes in the Q₁(0,0) region. These are paralleled by much-larger relative changes of the absorption in the region near 540 nm associated with the Q₁ transition of pheo a₁. The difference of the absorption spectra collected before and after continuous wave (CW) illumination at 1.7 K reveals changes in transitions that occur due to the illumination. Figure 3 shows a series of difference spectra taken at 1.7 K with illumination at 630 nm. The fluences used to obtain the difference spectra of Figure 3 span 3 orders of magnitude, ranging from 16 μW/cm² for 10 s (160 μJ/cm²) to 4.9 mW/cm² for 33 s (162 μJ/cm²).

An analysis of difference spectra obtained after high- (270 K) and low-temperature (1.7 K) illumination of PSII core complexes has assigned the prominent derivative feature
as mentioned previously, efficient Q A was observed with laser wavelengths of < 675 nm. However, no persistent spectral hole-burning was observed in Figure 3. No persistent spectral hole-burning was conducted with a laser power of 3.5 mW/cm² for 60 s. The bottom trace shows Q A ² formation, following 630-nm illumination with comparable burn fluence to the hole-burned spectra. Hole-burning with PSII in the S₁(Q A ) state results in a persistent spectral hole superimposed on the structure associated with electrochromic shifts due to Q A⁻ formation. With PSII in the S₁(Q A ) state, difference spectra after laser illumination at ~683 nm display persistent spectral hole-burning features only, because all PSII centers have been converted to the S₁(Q A ) state.

The series of spectra in Figure 3 establish that the illumination-induced changes do not lead to significant chl a radical formation. Any such radical formation would lead to the difference spectra displaying a net decrease in absorption (not conservative) in the chl a region. We find that the absorption differences are conservative to within 0.05 chl a per PSII core complex. Although initial Q A⁻ formation is more efficient for lower fluences, the shapes of all illumination-induced difference spectra are independent of illumination fluence and wavelength in the 500–700 nm range. At much-higher fluences (>10 J/cm²), sample damage can occur. The overall quantum efficiency of low fluence (<20 mJ/cm²) and low-temperature Q A⁻ formation has been estimated as ~10%, which is in agreement with the data in Figure 3.

3.2. Broadband Spectral Hole-Burning. Figure 4 shows the effects of broadband (2–3 cm⁻¹ width) laser excitation of a PSII spinach core complex poised in the S₁(Q A ) state and also in the photoreduced S₁(Q A⁻) state. The hole-burned spectra are the differences of the absorption spectra in the Q₁(0,0) region before and after laser illumination. Persistent spectral hole-burning was conducted with a laser power of ~3.5 mW/cm² for 60 s at 683.3 nm for the spectra in Figure 4. The hole-burned spectrum of a sample poised in the S₁(Q A ) state displays a spectral hole superimposed upon a background with considerable structure. This structured background is due to the Q A⁻ shift, as seen in Figure 3. No persistent spectral hole-burning was observed with laser wavelengths of ~675 nm. However, as mentioned previously, efficient Q A⁻ formation occurs for excitation at higher energies.

To perform measurements on PSII in the S₁(Q A⁻) state, a sample in the S₁(Q A ) state was illuminated with laser light at 630 nm with a similar fluence to that used for hole-burning (above). Subsequent illumination at 683.3 nm results in a spectral hole, resonant with the laser frequency. This is formed with no background changes associated with Q A⁻ formation as the 630 nm pre-illumination converts the sample quantitatively to the S₁(Q A⁻) state. The photoproduction associated with hole formation is found on both sides of the resonant hole, and its distribution reflects that of the absorption feature at 683.5 nm. Figure 5 shows the absorption spectra of syn. 6803 (top, thick line), and spinach (bottom, thick line) PSII core complexes in the S₁(Q A ) state at 1.7 K. Hole-burned spectra show persistent spectral holes burnt with a broadband laser (line width of 2–3 cm⁻¹) in syn. 6803 (top, thin line) and spinach (bottom, thin line) PSII cores at 1.7 K. Hole depths (ΔA/A × 100%) are ~7.5% and ~10%, respectively, burnt with ~1.4 mW/cm² for 10 s, and ~3.5 mW/cm² for 60 s, respectively. The asterisk (*) indicates laser-induced hole-filling of a previously burnt hole.
by the shaded regions. This may be due to the larger number of chl molecules in the \( S_1(\text{Q}_a) \) state before illumination. Hole-burned spectra show the different phonon structure (sidehole to lower-energy of 690.1 nm hole) of holes burnt with a broadband laser (line width of 2–3 cm\(^{-1}\)) at 683.4 and 690.1 nm in a spinach PSII core in the \( S_1(\text{Q}_a) \) state at 1.7 K. The 690.1 nm hole (right) has been multiplied by a factor of 7 (\( \times 7 \)). The hole depths are \( \sim 17\% \) (left) and \( \sim 38\% \) (right), attained with burn fluence of \( \sim 15 \text{ mW/cm}^2 \) for 5 min.

Figure 6. Absorption spectra (thick line) at 1.7 K of a spinach PSII core complex, in the \( S_1(\text{Q}_a) \) state before illumination. Hole-burned spectra (thin lines) show the photoproduct distribution of persistent spectral holes burnt (broadband laser line width of 2–3 cm\(^{-1}\)) in a spinach PSII core in the \( S_1(\text{Q}_a) \) state at 1.7 K, with \( \sim 3.5 \text{ mW/cm}^2 \) for 20 s. All hole depths are \( \sim 9\%–10\% \). This sharp photoproduct is superimposed on a broader underlying distribution, which is indicated by the shaded regions.

The overall characteristics of the action spectra are similar for all our PSII preparations. Laser illumination at wavelengths of \( < 670 \text{ nm} \) was almost completely ineffective in hole formation. There is a narrow SDF centered at \( \sim 684 \text{ nm} \) that dominates the action spectra. From simple Gaussian fits, the prominent feature in the spinach core action spectrum is located at 683.8 nm with a width (fwhm) of 40 cm\(^{-1}\), whereas that of \( \text{syn. 6803} \) is centered at 683.6 nm with a width of 75 cm\(^{-1}\). This difference may be attributed to differing inhomogeneous spectral distributions of the chromophore(s) that results in hole-burning in this region.

There are chromophores to both higher and lower energy of the narrow SDF that contribute to the action spectrum, extending...
from 675 nm to 695 nm. The higher-energy component corresponds to hole-burning by a small fraction of the chromophores absorbing in the Q_(0,0) region. However, at wavelengths of ~686 nm, where overall absorption is relatively weak, hole-burning is highly efficient, and hole depths of ~50% can be achieved.

Spectral holes can be filled either by warming the sample to >30 K or by illumination of the sample at wavelengths of ~670 nm. Systematic measurements with PSII in the S_(1)(Q_A) state are more difficult than those for the S_(1)(Q_(A^-)) state, because the laser illumination used in hole-burning leads to efficient Q_A^- formation. This results in the electrochromic shifts as seen in Figures 3 and 4, obscuring features that were solely due to hole-burning. In addition, it is not feasible to regenerate the S_(1)(Q_A^-) state of a PSII sample quantitatively. Warming the sample to >150 K only partially regenerates the sample. Annealing the sample to 270 K does lead to full Q_A^- oxidation; however, when a sample is subsequently re-glassed, there can be subtle changes that make accurate comparisons of spectra previously obtained difficult.

By performing hole-burning measurements as a function of laser fluence, while also monitoring Q_A^- formation via broadband spectral changes (as in Figure 3), we were able to make estimates of hole-burning efficiencies for spin-allowed PSII core complexes in both the S_(1)(Q_A^-) and S_(1)(Q_(A^-)) states over a range of wavelengths. Quantum efficiencies of persistent spectral hole-burning were as high as 10^-2 with PSII in the S_(1)(Q_A^-) state and 10^-3 in the S_(1)(Q_(A^-)) state. Laser burn fluences used in this work were as low as 50 nJ/cm² and, for high-resolution measurements, typically on the order of 1–10 μJ/cm². This is orders of magnitude lower than the fluences commonly used in hole-burning.50,51 and in two recently reported crystalline chromium systems that display initial nonphotochemical hole-burning quantum efficiencies of ~10^-3–10^-2.52,53 Our hole-burning fluences are comparable to, and typically lower than, those used in amorphous systems that display the most-efficient nonphotochemical hole-burning.54,55 Burn fluences utilized in hole-burning on photosynthetic antenna systems are also orders of magnitude higher than those used in this work (see, for example, hole-burning in the Fenna–Matthews–Olsen Antenna complex56). A spectral hole with a depth of 1.6% could be obtained near 684 nm in the Q_(0,0) absorption spectrum of a spinach PSII core in the S_(1)(Q_A^-) state with defocused light from a 0.75 m monochromator/tungsten lamp system operating with 50-μm slit widths for 7 min. This corresponds to a fluence of ~400 nJ/cm².

Hole-burning in PSII samples in the S_(1)(Q_A^-) state is invariably accompanied by Q_A^- formation, as determined by broadband difference-spectra (as in Figure 1). These processes seem to have very comparable efficiencies. Q_A^- formation was observed following excitation in the 683-nm region but also following excitation in the 685–695 nm range. Prolonged low-intensity (<1 mW/cm²) illumination in the range of 685–695 nm leads to full (approaching 100%) and efficient Q_A^- formation, accompanied by relatively deep spectral holes. In a control experiment, relatively intense excitation (100 mW/cm² for 10 min) at 750 nm led to no observable Q_A^- formation.

Q_A^- formation can only occur as a direct consequence of charge separation in P680*. Therefore, we are left with the surprising observation that either P680 absorbs directly in the 685–695 nm range or is excited by excitation transfer from other pigments that are also absorbing in this range. The remarkably high efficiency of hole-burning, particularly in the S_(1)(Q_A^-) state, suggests that the hole-burning mechanism is associated with P680 excitation and subsequent charge separation. Absorption in this region has been associated with a well-known trap pigment in CP47 with a broad band whose maximum is near 690 nm.55 The lowest-energy states of CP43 are relatively sharp and absorb near 683 nm.13 These observations are given further consideration in the discussion section.

3.4. High-Resolution Spectral Hole-Burning. The previously described broadband experiments were performed with a dye laser operating at a relatively broad line width of 1–3 cm⁻¹. This allowed spectral changes over a very large wavelength range (440–750 nm) to be monitored with high sensitivity, using a conventional spectrometer operating with a spectral resolution comparable to the laser line width. To measure homogeneous holewidths, experiments with the much-higher resolution of ~20 MHz (~0.001 cm⁻¹) were performed using the diode–laser system described in the Materials and Methods section. Following hole-burning at a specific wavelength, the laser was attenuated and repeatedly scanned over ~30 GHz (1 cm⁻¹). Spectral holes were measured in transmission, which is an approach that allows much-lower readout fluences than necessary in fluorescence excitation experiments.

Measurements were performed on PSII spinach cores in the S_(1)(Q_A^-) state by pre-illumination of dark-adapted samples using 632.8 nm radiation with ~2 mW/cm² for ~5 min. Hole-burning on PSII cores in the S_(1)(Q_A^-) state was performed on samples annealed in the dark at 260–270 K for 5 min. Care was taken to ensure that the sample was kept dark before exposure to the laser at the burn wavelength, ensuring that contamination of the sample by PSII in the S_(1)(Q_(A^-)) state was insignificant. All hole profiles were well-described as a single Lorentzian. The delay between burn completion and hole readout was typically...
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<30 s and not greater than ~2 min. The hole depth can decay slightly (~15%) over a time period of ~2 min but negligible hole-broadening occurred during this time.

3.5. Hole-Burning Widths in S1(QA) and S1(QA⁻). Figure 9 shows shallow persistent spectral holes burnt at 685.20 nm in the Qy(0,0) band in a spinach core sample in the S1(QA) (bottom) or S1(QA⁻) (top) states. The hole depths are 4.7% and 1.5%, respectively. This burn wavelength is similar to the maximum of the hole-burning action spectrum in Figure 8. The fluence used for these holes was 67 nW/cm² for 60 s (4 mJ/cm²). The signal-to-noise ratio of hole readout could be improved by extended averaging but was limited to ~50 scans to minimize the effect of temporal hole decay and laser-induced hole-filling. The contribution to the measured holewidth due to spectral diffusion was evaluated by varying the delay period between burn completion and hole readout from a few seconds to a few minutes. On the time scale of ~2 min for hole readout, spectral diffusion was small and within the holewidth variation of 0.5 GHz.

Figure 9 immediately indicates that hole-burning was ~5 times more efficient in the S1(QA⁻) state, compared to the S1(QA) state. The holewidth was ~2 times broader, at 7.0 ± 0.3 GHz, compared to 3.6 ± 0.1 GHz in the S1(QA⁻) state. The hole-burning efficiencies observed with the diode—laser system were fully consistent with parallel experiments performed via broadband laser hole-burning and monochromator readout. Quantitative determinations of hole-burning efficiencies are made in a following section.

3.6. Excited-State Lifetimes. The dephasing processes associated with an electronic excitation determine the homogeneous line width of the zero-phonon line of an optical transition in the condensed phase. The dephasing time (T₂) has contributions from the excited-state lifetime (T₁) and a “pure dephasing” time (T₂*). The pure dephasing process results from fluctuations of a single-site transition frequency induced by time-dependent interactions of the chromophore with its environment. The homogeneous line width (Γ hom) is given by

$$\Gamma_{\text{hom}} = (\pi T_{\text{hom}})^{-1} = (2\pi T_{\text{hom}})^{-1} + (\pi T_{\text{deph}})^{-1}$$

In a hole-burning experiment where the laser line width is much less than the measured holewidth, this holewidth is twice the homogeneous line width (Γ hom = 2Γ hom). This is due to hole-burning involving at least two photons: one to burn and another to read the hole. The lifetime-limited holewidth, Γ hol = (πT₁)^{-1}, can be determined by extrapolating the measured holewidth to zero burn fluence and zero temperature, assuming the holewidth contribution due to T₂* vanishes. This process yields the lifetime-limited homogeneous line width and, consequently, a measure of the excited-state lifetime for the hole-burned chromophore.

The contribution from pure dephasing to the holewidth in spinach cores was determined by measuring the holewidth, extrapolated to zero fluence, at three wavelengths over the temperature range 2.5–10 K (Figure 10). The zero-fluence holewidths in the temperature range of 2.5–6 K can be fitted to the T⁻¹.3 temperature dependence commonly used for chromophores in amorphous hosts and the extrapolation to 0 K thus determined. The lifetime-limited holewidths obtained by a fit of T⁻¹.3 dependence were 2.0 ± 0.5 GHz at 684.75 nm, 1.5 ± 0.5 GHz at 685.20 nm, and 0.5 ± 0.3 GHz at 691.62 nm. The corresponding excited-state lifetime estimates are 160, 210, and 640 ps, respectively.

Boxer et al. provided the first report of nonphotochemical hole-burning in a well-defined protein matrix, along with the temperature dependence of the holewidth in the temperature range of 1.35–2.5 K. The hole readout was conducted in fluorescence excitation, and the width followed a T⁻¹.3 temperature dependence. A T⁻¹.3 temperature dependence has also been ascribed to the homogeneous holewidth in non-O₂-evolving PSII reaction centers isolated from spinach via fluorescence-detected hole-burning experiments. In these latter experiments, the T₁ lifetime of chromophores absorbing maximally near ~681 nm was 4 ± 1 ns, which approaches the chl a radiative lifetime of 8 ns. A T⁻¹.3 temperature dependence has also been reported for the Fenna–Matthews–Olsen antenna complex. Hole-burning efficiencies in all the aforementioned reports seem to be much lower than those observed in this work, and, consequently, higher burn fluences (>100 mJ/cm²) were necessary.

3.7. Hole-Growth Kinetics and Hole-Burning Quantum Efficiency. The initial hole-burning quantum efficiency (φ) can be estimated from the hole-growth kinetics. Typical kinetics for holes burnt with PSII in the S1(QA⁻) state are shown in Figure 11. The dotted line through the data is provided as a visual aid only. The quantum efficiency is calculated from the ratio of the number of absorbing centers excited to the number of photons absorbed, while accounting for those chromophores.
that undergo a change following excitation. The initial hole-burning efficiency is given by\(^\text{52,53}\)

\[
\phi = \frac{N_A c(\Gamma_h) d[\Delta A(A_0,dr)]}{[\Omega/(hv)](1 - 10^{-5})}
\]

where \(N_A\) is Avogadro’s number, \(c(\Gamma_h)\) the effective concentration of absorbing centers that are excited by the laser (given in units of mol/cm\(^3\)), \(d\) the optical path length (given in centimeters), \(\Omega\) the laser irradiance (given in units of W/cm\(^2\)), \(h\) Planck’s constant, \(\nu\) the laser frequency (in Hertz), \(A_0\) the pre-burn absorption at \(\nu\), and \(\Delta A/d\tau\) the slope of the \(|\Delta A|\) versus burn-time kinetic curve. In Figure 11, we have plotted \(|\Delta A|\) versus burn fluence, which is proportional to the burn time.

The initial hole-burning efficiency is determined by \(\Delta A/d\tau\) at \(\tau = 0\). When the burn fluence is reduced to a range in which the hole-growth kinetics are linear, the value of \(\Delta A/d\tau\) is given by the slope and no assumption about the overall functionality of the growth kinetics needs to be made. For the lowest burn fluences (<1.2 mJ/cm\(^2\)), the hole-growth kinetics are approximately linear (see inset in Figure 11). A lower limit for the initial hole-burning quantum efficiency can be made from these data. Independent data sets measured on individual samples with laser irradiances of \(\sim 7500\) and \(\sim 60\) nW/cm\(^2\) were determined. The hole-growth curves were observed to be independent of irradiance in this range.

The hole-burning experiments performed at 685.20 nm on spinach PSII core complexes probe the absorption feature at 683.5 nm with a full width at half maximum (fwhm) of \(\sim 50\) cm\(^{-1}\). The pre-burn absorption at \(\lambda = 685.20\) nm of the samples used was typically \(A_0 \approx 0.38\). Using the effective line width of 2.0 GHz determined in the previous section, we calculate the effective concentration of absorbing PSII centers to be \(\sim 2.0\times(30 \times 50) = 1.33 \times 10^{-7}\) of the total PSII concentration. The PSII concentration was \(2.90 \times 10^{-3}\) mol/cm\(^3\) in this work. This gives the effective concentration of excited chromophores within one homogeneous line width to be \(3.86 \times 10^{-12}\) mol/cm\(^3\) of PSII cores.

From the data in Figure 11, the initial hole-burning efficiency in the \(S_1(Q_A^-)\) state is estimated to be \(\sim 2 \times 10^{-3}\). The hole-burning kinetics exhibit a strongly dispersive behavior, with the rate of hole-burning decreasing significantly for longer burn times. Measurements of hole-growth kinetics on PSII in the \(S_1(Q_A)\) state are complicated by the creation of PSII particles in the sample, which are in the \(S_1(Q_A^-)\) state. Such experiments were not pursued in this work. However, from the relative hole areas in Figure 9 and corresponding hole-burning data obtained via broadband laser excitation, hole-burning in this state is \(5-10\) times more efficient than that for PSII in the \(S_1(Q_A^-)\) state and approaches a value of \(10^{-2}\).

4. Discussion

Hole-burning and hole-burning action spectra have been reported for D1/D2/cyt\(c_{559}\) reaction-center preparations\(^\text{36,45}\) and for isolated CP43\(^\text{43}\) and isolated CP47\(^\text{44}\) proteins prepared from spinach. Each of these core fragments exhibit persistent spectral hole-burning with SDFs near 684 nm. However, there are clear differences in the fundamental characteristics of the hole-burning processes presented in this work, compared to those reported for CP43, CP47, and D1/D2/cyt\(b_{559}\) fragments.

4.1. Hole-Burning Efficiencies. PSII core complexes exhibit hole-burning efficiencies that are orders of magnitude higher than those observed in core fragments. Consequently, much-higher laser fluences (typically \(>100\) mJ/cm\(^2\))\(^\text{42-45}\) are required in hole-burning experiments on the isolated fragments. The hole-burning efficiency observed for spinach PSII cores in the \(S_1(Q_A^-)\) state approaches \(10^{-2}\) and, thus, is comparable to the efficiency of \(S_1(Q_A^-)\) formation.\(^\text{14}\) This exceptional hole-burning efficiency is present in a system exhibiting lifetimes as short as 40 ps. We have associated this remarkably high efficiency with the primary charge separation of P680\(^*\). Burn efficiencies are observed to be up to an order of magnitude lower for PSII in the \(S_1(Q_A^-)\) state. This indicates that the primary charge separation in PSII is less efficient in this state. It has been suggested that, in the presence of the negatively charged Q\(^{-}\) anion, the charge separation of P680\(^*\) is inhibited. The formation of Pheo\(D^{1+}\) is significantly less energetically favorable in this situation.\(^\text{25}\)

4.2. Hole Structure: Sideholes and Photoproducts. Broadband hole-burning spectra in PSII cores are very distinct to those observed in the isolated core fragments CP43, CP47, and D1/D2/cyt\(b_{559}\). These latter systems, hole-burning results in broad sideholes. The frequencies of the sideholes are relatively independent of the burn frequency. No such sidehole features are present in active PSII cores, and no hole-burning is observed with excitation above 676 nm. This wavelength is close to the center of the PSII core Qb\((0,0)\) absorption envelope. Laser excitation of isolated core fragments results in resonant hole-burning for burn frequencies that extend over most of the absorption envelope.

The photoproduct associated with hole-burning in PSII cores is also very distinctive in that it is distributed across the entire SDF and not characteristically shifted to higher energies. A blue-shifted photoprodut is evident in isolated CP43\(^\text{20,43}\) and is typical of many other systems that display nonphotochemical hole-burning.\(^\text{50}\)

The intensity of phonon sideband holes (PSBHs) observed in PSII cores with excitation near 684 nm is very weak compared to that reported for CP43,\(^\text{20,43}\) CP47,\(^\text{44}\) and D1/D2/cyt\(b_{559},^\text{45}\) in which PSBHs are observable. PSBHs build to the blue side of the resonant zero-phonon hole (ZPH), whereas pseudo-PSBHs build to the red side of the ZPH (see, for
example, ref 48). The electron–phonon coupling of isolated core fragments is weak, with Huang–Rhys factors of $S \approx 0.25$ for isolated CP43, $S \approx 0.2$ for the 690 nm state of isolated CP47, and $S \approx 0.6–1.1$ for D1/D2/cytb559. In the short-burn time limit, $e^{-2S}$ is a reasonable approximation to the ratio of the integrated intensity of the ZPH to that for the (ZPH + pseudo-PSBH). The data in Figure 7 then clearly show that the Huang–Rhys factor ($S$) for the $-684$-nm SDF in PSII core complexes is remarkably small.

### 4.3. Excited-State Lifetimes

The decay of the excited state, and many alternative processes, can broaden spectral holes. Consequently, excited-state lifetimes as determined by hole-widths in hole-burning spectroscopy provide a lower limit to their actual value. By comparison, pump–probe methods may overestimate excited-state lifetimes, because of technical limitations in fast response systems. Persistent spectral hole-burning favors the observation of long-lived excited states as holewidths become narrow and far easier to detect and characterize.

At low temperatures (<10 K), PSII cores do not emit strongly out of narrow band state(s) near 683.5 nm. The dominant emission of PSII cores is broad, peaking near 695 nm. This broad emission can be attributed to a chlorophyll a in CP47. This assignment is supported by the observation that low-temperature emission spectra of PSII cores and isolated CP47 in this spectral region are both similar to that of D1/D2/cytb559/CP47 particles. PSII cores emit with a similar quantum efficiency as isolated CP47.

Isolated CP43 particles emit in a relatively narrow region near 683 nm at low temperatures, and also with good quantum efficiency (~10%). Several experiments have indicated that emission has been identified as originating from two states (labeled A and B), both absorbing in a narrow but composite band near 682 nm. Hole-burning experiments established a radiative lifetime-limited holewidth of ~40 MHz in the A state.

Low-fluence, narrow-band hole-burning has been observed in D1/D2/cytb559 preparations, also providing line widths for hole-burning near 682 nm that correspond to radiative lifetimes of chlorophyll a (~8 ns). The action spectrum extends over a very broad range, from 660 nm to 690 nm.

Our hole-burning experiments establish the existence of a composite SDF in both spinach BBYs and PSII cores as well as in syn. 6803 PSII cores. All SDFs have a narrow peak near 684 nm. The lifetime of the excited-state in this region in spinach cores in the $S_1(Q_A)$ and the $S_1(Q_X)$ states were determined to be $\geq 160$ and $\geq 40$ ps, respectively. We have suggested that CP43 is not responsible for the entire absorption peak at 683.5 nm in spinach cores, and that P680 absorbs strongly in this region. Having determined homogeneous holewidths from measurements, it seems unlikely that the hole-burning observed in this work can be attributed to direct excitation of—and, thus, subsequent hole-burning in—P680*. If this were to occur, it would constrain the lifetime of P680* to be $\geq 40$ ps, which is more than 10 times longer than the generally accepted value at low temperature (see Introduction). As mentioned in the Introduction, photon echo experiments have been modelled to show strongly dispersive charge-separation kinetics. The range of rates was from (1.5 ps)$^{-1}$ to $\sim$1 (ns)$^{-1}$ in D1/D2/cytb559 preparations at 1.3 K. The hole-burning observed in our samples cannot be easily attributed to burning of the lowest-energy reaction center transition of a small fraction of PSII centers having long charge-separation times. First, deep spectral holes remain narrow, and, thus, a majority of the reaction centers would need to have charge-separation lifetimes of >40 ps. Second, the SDF (see Figure 8) shows that two electronic states contribute to hole-burning. Higher-lying excitonic components of the reaction center would relax within <1 ps and, thus, would not result in narrow spectral holes.

We propose that the state(s) in CP43 that cause fluorescence in the isolated protein transfer excitation to P680 in $\geq 40$ ps in the intact core and the subsequent hole-burning in PSII is attributed to a mechanism that involves charge separation in P680*. The rate of excitation transfer from the CP43 fluorescent states to P680 when PSII is in the $(Q_A^\pm)$ state would be $\geq 160$ ps.

Our holewidth measurements suggest a CP43 state in intact PSII cores that has a lifetime of $<160$ ps. Thus, radiative emission from such a “slow transfer” state in CP43 would be weak ($\geq 160$ ps/\~8 ns, or <2% quantum efficiency). This is consistent with PSII core emission spectra that show only weak emission in the 683 nm region. The spectral position of the CP43 slow transfer states in syn. 6803, as inferred from hole-burning action spectra, is located at slightly higher energy than that in spinach. We have reported that the strong negative low-temperature CD feature observed in spinach PSII cores at 683.5 nm is split in syn. 6803 into two features, at 681.1 and 683.8 nm. We had tentatively assigned the lower-energy feature to P680 and the higher-energy feature to CP43. On current evidence, this assignment would be inverted, with the lower-energy component being associated with the slow transfer state in CP43 in syn. 6803.

### 4.4. The Lowest-Energy Excited State of P680

Our absorption and hole-burning experiments are performed within the temperature range of 1.7–10 K and with irradiances as low as 10 nW/cm$^2$. Sample heating, particularly at the lowest irradiances used, is negligible. Implicit in an assignment of slow transfer states in CP43 that results in hole-burning induced by charge separation in intact PSII, is the presence of an excited state of P680 at an accessible energy. At 1.7 K, the thermal Boltzmann energy $(kT)$ is only 1.2 cm$^{-1}$. Thermal activation beyond $5kT$ (6 cm$^{-1}$ at 1.7 K) becomes increasingly unlikely. We are forced to conclude that P680 must be a lower energy than the 684 nm band in both spinach and syn. 6803. The requirement that the lowest excited state of P680 be at a lower energy than 14 620 cm$^{-1}$ (684 nm) is enforced by our observation of complete and efficient $Q_A^\pm$ formation.

We propose that a broad absorption of P680 extends to lower energy and underlies the CP47 absorption in this region. This proposal can account for the very high efficiency and deep absorption of P680 in the 695 nm region. The requirement of this state is ~600 ps for PSII in the $S_1(Q_A)$ state and is ~4 times longer than that of the corresponding CP43 slow transfer state. A longer lifetime of the CP47 slow transfer state is consistent with a ~5% emission quantum efficiency of PSII cores at 4 K. Further evidence is supporting this proposal. The CP47 slow transfer state occurs to the lowest-energy state of P680.

These arguments allow for the possibility of efficient excitation transfer from both CP43 and CP47 slow transfer states to P680 with high efficiency (up to 90%). P680 excitation results in both efficient $Q_A^\pm$ formation when a PSII sample prepared in the $S_1(Q_A)$ state is excited and efficient hole-burning for samples in either the $S_1(Q_A^\pm)$ or $S_1(Q_X)$ states. The emission efficiency of PSII cores at room temperature is known to be ~3 times lower when $Q_A$ is reduced (the closed state) than when
Q_A is oxidized (the open state). Unless low-temperature emission experiments are made at extraordinarily low fluence (<5 mJ/cm²), spectra will invariably be recorded for the equivalent of Sn(Q_A⁻) state.²⁵ Emission excitation illumination will rapidly photoconvert an Sn(Q_A) sample to the S1(Q_A) state.

The lowest-energy excited state of bacterial reaction centers is known to be significantly broader than higher states (see, for example, Mar²⁶). Therefore, a correspondingly broad lowest-energy excitation of a primary donor chromophore is not without precedent. The origin of this broadening has been the subject of ongoing discussion.⁶⁴–⁶⁶ The energy spacing between the narrow higher-energy P680 band near 684 nm and a putative broader band near 690 nm is ϰ10 cm⁻¹ and, thus, is well within the range suggested by dipole–dipole couplings between closest interpigment distances of the D1/D2 chl a and pheo a molecules.³–⁵,⁶⁷

Our analysis of band areas in PSII cores¹ suggested that the low-energy tail was reasonably well accounted for by one (CP47) chl a. We further estimate that any underlying absorption is likely to have an area corresponding to ϰ0.2 chl a. This indicates that a dominant P680 coupling may be present between parallel transition dipoles. This arrangement would result in a weak lowest Davydov (exciton) component, as is observed in some dye aggregates.⁶⁸ Such an arrangement is different from the situation in the special pair in bacterial reaction centers, where the strongest coupling is between bacteriochlorophylls with approximately antiparallel transition dipoles. X-ray data¹–⁵ establish that the Q_a transition dipoles of P_D1 and P_D2 are also approximately antiparallel. However, transition dipoles of P_D0/Chl_D2 and P_D0/Chl_D1 pairs are close to parallel, and their molecular separation is only slightly greater than that of P_D0/P_D2. An assignment of a weak lowest-energy exciton component of P680 calls for a detailed investigation and further consideration.

4.5. Hole-Burning Mechanism. A widely applied model for nonphotochemical hole-burning⁶⁰ describes the chromophore potential-energy surface as a two-level system (TLS). Hole-burning results from tunneling between excited-state TLSs, and, consequently, the efficiency of hole-burning is related to the excited-state lifetime. As mentioned previously, the lifetime of our hole-burning states are much shorter than the fluorescence lifetime of chl a; therefore, conventional nonphotochemical hole-burning can be expected to be correspondingly less efficient. Because efficiencies of fluorescent pigments are typically only 10⁻³–10⁻⁵, an alternate hole-burning mechanism is required.

We propose that when P680⁺ charge separates, following excitation from a slow transfer pigment in CP43 or CP47, subtle changes in protein–pigment conformations occur. These changes may result from structural reorganizations of the protein and the transition energy of intercalated pigments may then be subtly altered. The transition energies of those chromophores directly excited via laser excitation will shift to slightly different values. The transition energies of chromophores not directly excited are not likely to be strongly correlated. The result is a spectral hole at the laser frequency with no sidehole features associated with other pigments. Significantly, the hole-burning efficiency now is not dependent on the lifetime of the slow transfer state, but rather on the probability that P680 excitation and charge separation ensues. We do not observe a large change in hole-burning efficiency between the 160-ps CP43 state and the 600-ps CP47 state for PSII cores in the S1(Q_A⁻) state. This supports a hole-burning mechanism that is independent of excited-state lifetime.

A “charge transfer” hole-burning mechanism is not without precedent. A similar mechanism, which involves charge separation between pairs of interacting metallophthalocyanines, has been suggested to explain the hole-burning observed for these chromophores trapped in frozen rare-gas matrices.⁶⁹,⁷⁰

4.6. P680 Charge-Separation Rate. Our observation of long-lived states in PSII cores, transferring excitation to P680 at low temperatures, does not provide support for the “shallow trap” model of P680. In this model, excitation equilibration between P680 and all antenna pigments is considered to be faster than the charge separation rate of P680. However, at physiological temperatures, the slow transfer states may be bypassed by thermally activated excitation transfer processes and, thus, may not be rate-limiting.

The absence of hole-burning at wavelengths of ϰ676 nm in active PSII, along with the absence of emission at these wavelengths, is consistent with the belief that excitation transfer from pigments absorbing at these higher energies is indeed faster than ϰ500 fs. The potential exists that direct low-temperature excitation of the lowest-energy excited state of P680 may result in hole-burning in this absorption, particularly for a PSII sample in the S1(Q_A) configuration. The holewidth here may provide the first direct measure of the rate of charge separation in native P680⁺. Such holes may be 30–300 GHz wide, corresponding to a charge-separation rate of 1–10 ps. They could be expected to be relatively shallow if the absorption of the lowest excited state of P680 is, indeed, weak. Such a hole would be observed in parallel with the sharp (0.5 GHz) CP47 holes reported here. We note that, in the bacterial reaction center, narrow-band excitation into the lowest-energy primary donor band leads to extremely broad spectral holes whose width does not relate to the charge-separation rate.

5. Conclusions

We have identified highly efficient persistent spectral hole-burning in the active photosystem II (PSII), which we associate with a charge-separation mechanism that involves native P680⁺. Lifetime-limited holewidths that have been determined to be in the 0.5–2.0 GHz range indicate the presence of long-lived excited-states in CP43 and CP47, which transfer excitation to P680 with good efficiency. The ensuing charge-separation process is held responsible for the high hole-burning efficiency observed.

Efficient hole-burning and electrochromic shifts associated with Q_A⁻ formation occur upon low-fluence excitation in the 685–695 nm spectral range. This is interpreted by proposing a weak, broad lowest excited state of P680 underlying the broad CP47 absorption in this range. At low temperatures, excitation of the “trap” CP47 in intact PSII leads to efficient charge separation. The “slow transfer” states in CP43 and CP47 may, indeed, serve a significant function in active PSII if these processes are rate-limiting at physiological temperatures.

References and Notes

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