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Optical Spectra of Synechocystis and Spinach Photosystem II Preparations at 1.7 K: Identification of the D1-Pheophytin Energies and Stark Shifts

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Abstract: We report and compare highly resolved, simultaneously recorded absorption and CD spectra of active Photosystem II (PSII) samples in the range 440−750 nm. From an appropriately scaled comparison of spinach membrane fragment (BBY) and PSII core spectra, we show that key features of the core spectrum are quantitatively represented in the BBY data. PSII from the cyanobacterium Synechocystis 6803 display spectral features in the Q_y region of comparable width (50−70 cm^{-1} fwhm) to those seen in plant PSII but the energies of the resolved features are distinctly different. A comparison of spectra taken of PSII poised in the S_1Q_A and $S_2Q_A^-$ redox states reveals electrochromic shifts largely attributable to the influence of Q_A^- on $Pheo_{D1}$. This allows accurate determinations of the $Pheo_{D1}$ Q_y absorption positions to be at 685.0 nm for spinach cores, 685.8 nm for BBY particles, and 683.0 nm for Synechocystis. These are discussed in terms of earlier reports of the $Pheo_{D1}$ energies in PSII. The Q_x transition of $Pheo_{D1}$ undergoes a blue shift upon Q_A reduction, and we place a lower limit of 80 cm^{-1} on this shift in plant material. By comparing the magnitude of the Stark shifts of the Q_x and Q_y bands of $Pheo_{D1}$, the directions of the transition-induced dipole moment changes, $\Delta\mu_x$ and $\Delta\mu_y$, for this functionally important pigment could be determined, assuming normal magnitudes of the $\Delta\mu$'s. Consequently, $\Delta\mu_x$ and $\Delta\mu_y$ are determined to be approximately orthogonal to the directions expected for these transitions. Low-fluence illumination experiments at 1.7 K resulted in very efficient formation of Q_A^-. This was accompanied by cyt b_{559} oxidation in BBYs and carotenoid oxidation in cores. No chlorophyll oxidation was observed. Our data allow us to estimate the quantum efficiency of PSII at this temperature to be of the order 0.1−1. No Stark shift associated with the S_1-to-S_2 transition of the Mn cluster is evident in our samples. The similarity of Stark data in plants and Synechocystis points to minimal interactions of $Pheo_{D1}$ with nearby chloropyll pigments in *active* PSII preparations. This appears to be at variance with interpretations of experiments performed with *inactive* solubilized reaction-center preparations.

Introduction

Photosystem II (PSII) is a pigment−protein complex embedded in the thylakoid membrane of plants, algae, and cyanobacteria. A key part of the electron-transfer chain of the photosynthetic light reactions, it utilizes solar energy to extract electrons from water and reduce quinones, resulting in the production of molecular oxygen and a proton gradient over the membrane (reviewed in refs 1,2).

In the PSII reaction center, the 10 membrane-spanning helices of the D1 and D2 subunits hold the redox-active cofactors and pigments in pseudo-C_2 symmetry. These are six chlorophyll (chl) *a*, two pheophytin (pheo) *a*, two plastoquinones (Q_A and Q_B), and a non-heme Fe ion. Two tyrosine residues, D1−161 (Y_Z) and D2−160 (Y_D) are redox-active, and on the lumenal side of the enzyme the water-oxidizing tetra-Mn cluster is held by the D1 protein with support from several Mn-stabilizing subunits. Two β-carotenes (car) are also present on the D2 side.[3] Despite the spatial pseudosymmetry of the system, only one branch is active in the catalytic electron flow through the enzyme. Closely associated with the D1 and D2 subunits is cytochrome (cyt) b_{559}, and the inner antenna proteins CP43 and CP47. Also present in the PSII assembly are several nonpigment proteins and the energy-trapping antenna complex. (For recent progress in structural determination, see refs 3−5.)

During physiological enzymatic turnover, light energy is absorbed by the antenna pigments and funneled into the primary reaction-center electron donor P680, which consists of one or more of the pigments bound to D1 and D2. P680* subsequently reduces the pheo bound to D1 ($Pheo_{D1}$). The charge-separated

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state $P680^+/Pheo_{D1}^-$ is stabilized when $P680^+$ is rereduced by Y_Z, which in turn rapidly oxidizes the Mn cluster. The Mn cluster extracts electrons from water in a four-step process called the S-cycle, during which it cycles through the $S_0 \cdots S_4$ redox-states. On the electron acceptor side, Q_A accepts the electron from $Pheo_{D1}^-$ and passes it on to Q_B, which after two such reductions is protonated and replaced.

Plant PSII has been studied in preparations of different degrees of integrity. The highly solubilized D1/D2/cyt b_{559} preparations (D1/D2/b559)[6] contain 5 or 6 chl and the two pheos, and are capable of primary charge separation and recombination. PSII core complexes retain the inner antenna CP43 and CP47 as well as the Mn cluster and the Mn-stabilizing protein OEC33, and perform Q_A reduction and oxygen evolution to some extent. PSII-enriched membrane fragments have all the Mn-stabilizing proteins intact, and also retain the LHC; this preparation is fully active and more stable than the cores.

To restrict the number of pigments contributing to the absorption envelope, many investigations of the reaction-center pigments have been performed on D1/D2/b559 material. We are currently undertaking a comparative study of a range of differently solubilized PSII complexes, including isolated CP43 and CP47 subunits. We have reported[7,8] substantial spectral differences between the (appropriately scaled) summation of spectra of isolated CP43, CP47, D1/D2/b559 and that of fully intact preparations. Further studies of the subunits are now being undertaken to detail the substantial changes between the spectra of component proteins and their contribution within the fully active PSII assembly.

These comparisons[7,8] suggest that data from D1/D2/b559 particles do not necessarily represent processes in the intact reaction center, making the use of PSII cores as a model system preferable. To this end, we use a plant PSII core preparation developed in our lab,[7] which (i) maintains quantitative oxygen-evolving activity from the parent PSII membrane fragments, (ii) has very similar pigment composition to that found for the PSII crystals,[3,4] and (iii) exhibits unprecedented spectral detail, particularly in the chl Q_y region. To further ensure the relevance of our results to the intact photosystem, we employ protocols which minimally disturb the samples. By using a combination of cryoprotectants, we keep the concentration of each glassing agent to a minimum. As far as possible, we avoid the use of external reactants (such as reductants and oxidants) with the sample, and focus on the charge-separated states formed naturally in the enzyme upon illumination. To avoid photodamage of the samples, we illuminate over brief periods of time using filtered light. Finally, to confirm that our results do reflect intrinsic characteristics of the functioning photosystem, we perform parallel measurements on PSII cores and the more native PSII membrane fragments (BBY), using both optical and EPR techniques.

With the X-ray crystal structure of cyanobacterial PSII now available,[3,4,9−11] it is of great relevance to establish the extent to which the cyanobacterial and plant-derived PSII may be considered identical at the functional-spectroscopic level. The two forms of PSII exhibit some differences in protein composition: plant PSII is connected to the membrane-bound, chlorophyll-containing LHCII, whereas cyanobacterial PSII utilizes phycobilisomes, phycocyanin-containing proteins connected to PSII on the stromal side, for energy trapping. In plants, the Mn cluster is stabilized by OEC33 and the 16- and 23-kDa proteins, while the cyanobacterial OEC is protected by OEC33, the 12-kDa subunit, and cyt c_{550}. Although the catalytic electron-transfer chain seems to function similarly in the different species,[12] some differences in pigment and cofactor behavior have also been noted. In accordance with Hankamer et al.,[13] we have determined that plant PSII cores contain 7 β-car per 32 chl *a*,[7] whereas the equivalent number reported for Synechocystis is 14 β-car per 38 chl *a*.[14,15] Furthermore, low-temperature illumination of plant PSII has been reported to induce two different chl radical species, while cyanobacterial PSII only showed one such species.[14,15] The EPR signals from the Mn cluster also show slightly different characteristics in the two systems.

The nature of the pigments at the heart of PSII—the four central chl and two pheo molecules—remains unclear. They are all approximately 10 Å (center-to-center) from their nearest neighbor, a distance which allows for weak excitonic coupling ($\sim$100 cm^{-1}). The exact coupling scheme, which may hold the key to the unique functional properties of P680, is still unknown. A definitive interpretation of the large body of spectroscopic data available is made difficult by the spectral congestion in the chl Q_y region, and proposed models of the nature of P680 have ranged from interpretations assigning monomeric chl and pheo pigments[16] to an exciton coupled system involving the majority of the pigments.[17,18]

We have proposed[7,8] that the prominent, sharp (50 cm^{-1} fwhm) feature in plant core complexes, with an intensity of 2.2 chl at 683.5 nm, and a weaker excitonic partner 187 cm^{-1} to higher energy, are associated with P680 in *fully functional* PSII. This was based on CD, magnetic CD (MCD) and illumination-induced Stark-shift results. Isolated, solubilized CP43 and CP47 complexes have been extensively studied.[19−27] They also display spectral features in this region but CP43 in particular[21] has a

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composite feature near 683 nm, assigned to two isolated chl trap states. However, as indicated above,[7,8] well-resolved features in active core complexes are not well accounted for by features associated with isolated CP43 and CP47. We note that the spectral properties of these proteins in the solubilized fragments may well be modified upon assembly of the active core complex. These relationships are considered in the discussion, in the light of results presented here.

One method of identifying individual excitations in the crowded Q_y spectral region is to place a charge on a known cofactor in the reaction-center region, and monitor the electrochromic effects on the nearby chromophores resulting from this charge. Specifically, reduction of Q_A has a significant electrochromic effect on $Pheo_{D1}$, situated only 12−13 Å away.[3,4] (The localization of a negative charge on Q_A following charge separation has been established by EPR spectroscopy.[28,29]) Charged species can be created in a straightforward way by illuminating PSII, thus poising the enzyme in different charge-separated configurations. Junge and co-workers[30] measured transient absorption differences of pea PSII cores at room temperature while exposing them to short flashes of light. This resulted in absorption difference patterns that were fitted to a model involving $Pheo_{D1}$ and three chl *a* species. Diner and co-workers[31] exposed Mn-depleted PSII from Synechocystis to a chemical reduction/illumination protocol to produce states with Q_A reduced and oxidized. The 77-K absorption spectra were measured before and after this treatment, and subtracted. Both studies showed features attributed to a blue shift of an unresolved low-energy absorption feature in the chl Q_y region, which was assigned to $Pheo_{D1}$. Shifts were also seen in the 550-nm region.

In the current report, we have used low-temperature (260-K) illuminations to produce the $S_2Q_A^-$ redox configuration in PSII membrane fragments and fully active cores from spinach, and on PSII cores from Synechocystis P6803 (Syn. 6803). The charge-separated states were monitored in parallel samples by EPR spectroscopy. We report absorption spectra at 1.7 K recorded before and after illumination. The spectral changes induced by Q_A reduction allow us to precisely identify the $Pheo_{D1}$ absorption wavelengths in these preparations, as well as quantify the electrochromically induced shifts. Our plant data are generally consistent with previously reported room-temperature data,[30] while providing considerably narrower line widths and thus more detailed information. Our results from Syn. 6803 PSII provide a comparison of light-induced charge separation to the chemically induced reduction of Q_A^-,[31] as well as allowing a direct comparison of absorption and CD spectra and electrochromic shifts in spinach and cyanobacterial PSII.

Below 110 K, illumination does not result primarily in manganese oxidation. Alternative electron pathways have been investigated for illumination temperatures as low as 6 K, of Mn-depleted systems (see e.g., refs 15,32). We have performed 1.7-K illumination of our Mn-containing preparations and monitored changes in the optical spectra in the 440−750 nm region.

Experimental Section

PSII Sample Preparation and Illumination Procedures. PSII membrane fragments were prepared from market spinach as described in ref 33 and stored at 12−16 mg chl/ml in a buffer containing 0.4 M sucrose, 10 mM NaCl, 15 mM $MgCl_2$ and 20 mM MES pH 6.0 (NaOH) at 77 K. This material was further purified to fully active PSII core complexes using the method recently described,[7] and stored at 1−3 mg chl/ml at −80 °C in a buffer consisting of 0.4 M sucrose, 20 mM $MgCl_2$, 90 mM $MgSO_4$, 5 mM $CaCl_2$, 0.3 mg/mL β-d DDM and 20 mM BIS-TRIS pH 6.5 (HCl). Synechocystis core complexes were prepared from a glucose tolerant, HT-3A histidine-tagged strain of Syn. 6803. The cells were grown and biochemical procedures for isolating thylakoid and core complexes undertaken following the procedures of Hillier et al.[34] Samples were stored in the elution buffer, consisting of 50 mM MES (pH 6.0), 400 mM sucrose, 10 mM $MgCl_2$, 20 mM $CaCl_2$, 0.04% β-DDDM, and 100 mM imidazole, at −80 °C until used. Each preparation was undertaken under dim green light at 4 °C.

For optical measurements, thawed PSII material was diluted to a final concentration of ∼1 mg chl/ml in the storage buffer and glassing medium, achieving a Q_y peak OD of ∼1. The glassing medium used was a 1:1 mixture of glycerol and ethylene glycol, added to a final concentration of 45% (v/v). 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was included in the membrane fragment samples to a final concentration of 100 μM. All sample handling was done in dim green light, and the final solution was left in absolute darkness for 5 min at room temperature before freezing into He(*l*). To produce the $S_2Q_A^-$ state, the sample was warmed to 260 K, illuminated for 5−10 s, and immediately reglassed. Illumination was performed with a 150-W quartz halogen lamp imaged on the sample through a 10-cm water heat filter and a filter stack passing 500−600-nm green light, resulting in 1−2 mW/cm^2 at the sample. The illumination temperature of 260 K was chosen instead of the conventional 200 K to avoid fogging of the sample upon reglassing. In cores Q_B is absent, resulting in stable Q_A reduction at 260 K; in BBYs DCMU was added to block $Q_A \rightarrow Q_B$ electron transfer.

To confirm the redox state of the optical samples, parallel samples were prepared in EPR tubes (inner diameter 3 mm). These were prepared at 1 or 3 mg chl/ml (BBY and cores, respectively) with 40% (v/v) ethylene glycol/glycerol mix present and 100 μM DCMU in the BBY samples (as described above). To obtain the $S_2Q_A^-$ state, they were illuminated with the same setup as used for the optical samples, for 5−10 s at 260 K. The EPR samples were frozen in $N_2(l)$ before being transferred to the He flow cryostat.

EPR Spectroscopy. X-band EPR experiments were carried out on a Bruker ESP300E spectrometer, as per ref 33.

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Optical Spectroscopy. The PSII solution was introduced into a strain-free, quartz-windowed cell assembly with a path length of 0.2 mm, which was mounted onto a specially designed low thermal mass sample rod. The rod was introduced into an Oxford Instruments Spectromag 4 cryostat through a helium gas lock, the latter fitted with quartz observation/illumination windows. Glasses of good optical quality were obtained by plunging the sample rod into the liquid helium, cooling from 300 to 4 K over 30−60 s. All illuminations were performed with the sample in the cryostat assembly. Illuminations at 260 K were performed by withdrawing the sample into the windowed helium gas lock, where it was allowed to warm to 260 K. After illumination through the window in the lock, the glass was reformed by repeating the rapid cooling procedure.

Absorption and CD data were collected *simultaneously* on a spectrometer designed and constructed in our laboratory.[35] It is based on a 0.75-m Spex Czerny−Turner single monochromator using a 1200-l/mm grating blazed at 500 nm, with a dispersion of 1.1 nm/mm. A series of checks established that when spectra were rescanned, the wavelength reproducibility was better than 0.002 nm. Our spectral comparisons were thus not influenced by wavelength jitter or drift in the monochromator and have noise of $<10^{-4}$ ΔA. In these critical respects, they are of high precision.

The spectra presented in this report were recorded with 50-μm slit widths (0.05-nm resolution) to minimize turnover induced by light incident on the sample from the monochromator, and thus produce spectra of PSII with Q_A fully oxidized. We found that 500-μm slit widths, which result in ~50 μW of light at the sample, caused a significant portion of the PSII samples to perform photoconversion during a single scan. (This was used to estimate the quantum efficiency of PSII, see Results and Supporting Information.)

Results

Spinach PSII Cores and Membrane Fragments: Absorption and CD. Figure 1 shows the absorption (a) and circular dichroism (b) in the chl Q_y region of PSII cores and membrane fragments, recorded at 1.7 K. The CD has been magnified 500 times compared to the absorption data. Our CD and absorption spectra are measured with more than adequate resolution, and detected simultaneously. The high performance of our instrumentation is evident in that the CD presented, which is not smoothed or filtered, shows minimal noise. Our spectral comparisons are also not affected by any small sample-to-sample, instrumental, or even scan-to-scan variations, and the risk of wavelength drift between the absorption and CD data is excluded.

The spectra in Figure 1 have been scaled per PSII, by adjusting the area of the Q_y absorption bands to a BBY:cores ratio of 172:33. This ratio of areas was determined as follows. Our core complexes contain 32 chl *a* per two pheo *a*, and our BBY contain 134 chl *a* and 62 chl *b* per two pheo *a*.[7] The dipole strength ratio of chl *b*/chl *a* is ~0.6.[36] Isolated pheo *a* has 0.7 times the dipole strength of chl *a* in Q_y,[36] but there is some evidence that this factor is reduced significantly in the environment of the D1/D2/b559 particles.[37] On the basis of this, we use a Q_y absorption-strength ratio of pheo *a*/chl *a* ≈ 0.5.

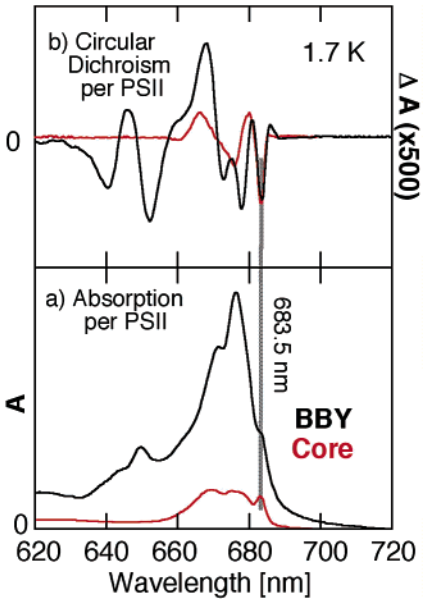

Figure 1. (a) Absorption and (b) simultaneously detected circular dichroism measured at 1.7 K of spinach BBY (black) and cores (red). The BBY data has been scaled per 172 chl *a* equivalents and the cores per 33 chl *a* equivalents, resulting in a per-PSII scaling (see text).

Assuming that each PSII contains two pheo *a*, this leads us to the ratio above, given in terms of effective chl *a* Q_y absorption units per PSII. (A variation of the pheo *a*/chl *a* Q_y ratio between 0.35 (suggested in ref 37) and 0.7 does not significantly affect these numbers.)

The scaled BBY absorption spectrum shows a chl *b* feature near 650 nm, some structure in the main chl *a* band, and a pronounced shoulder at 683.5 nm. The scaled core absorption data shows a complete absence of chl *b* (confirming previous HPLC analysis[7]), and detailed structure in the 670−690-nm region, a region which we propose is dominated by the reaction-center pigments.[7,8] The feature at 683.5 nm is present in the cores as well, and of comparable intensity as in the BBY. The band area of this peak corresponds to 2.2 chl *a* in total $Q_y(0,0)$ dipole strength.[7] The CD spectra have been scaled by the same factors as the absorption spectra. This per-PSII comparison identifies a quantitative retention of the negative CD feature at 683.5 nm from BBY and cores. The position, line width, and CD of the feature are thus unaffected by the purification procedure.

PSII Cores from Spinach and Synechocystis: Absorption and CD. A comparison of the 1.7-K CD and absorption in the Q_y region of plant and cyanobacterial core complexes is made in Figure 2. The areas in absorption of the two systems have been normalized, as it is known that both contain 32−35 chl *a* per core complex.[3,4,7] Both materials show a number of absorption features, with line widths of around 2−3 nm (50−75 cm^{-1}). An estimate of the line widths of the more prominent features were made from an analysis of the second derivatives of absorption spectra. A more complete least-squares Gaussian fitting analysis of the entire spectrum was not attempted, as the presence of 34 or more pigments, many of which have significant dipole−dipole (exciton) interactions affecting inten-

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Table 1. Positions of Spectral Features of 50−75 cm^{-1} Width from Spinach and Syn. 6803 PSII Core Spectra

		spinach cores		syn. 6803	
		feature[a] (nm)	Δ from 683.5 nm (cm^{-1})	feature (nm)	Δ from 684.1 nm (cm^{-1})
Q_y		683.5	0	684.1 (CD)[b]	0
		678.0	+118	684.2 (Abs)[c]	−2
		674.8	+188	678.0	+131
				670.0	+307
				666.0	+397
				665.8	+402
$Pheo_{D1}$ Q_y		685.0[d]	−32	683.0[d]	+23
$Pheo_{D1}$ Q_x	(Q_A)	546.1	shift (cm^{-1})	542.6	shift (cm^{-1})
	(Q_A^-)	544.3	+61	540.9	+58

[a] Unless otherwise specified, the feature positions were determined by second derivative analysis of the absorption spectra. [b] As determined from the CD feature in Figure 2. [c] As determined by subtracting the $Pheo_{D1}$ peak, deduced by fitting the shift, from the absorption spectrum. See Figure 7 and text. [d] As determined from the zero crossing of the shift, Figs. 5a and 6.

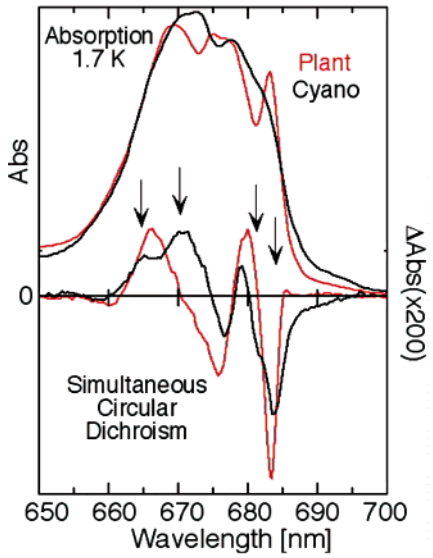

Figure 2. Absorption (left-hand scale) and simultaneously detected circular dichroism (right-hand scale) measured at 1.7 K on Syn. 6803 (black) and spinach PSII cores (red). The absorption spectra have been scaled to the same area (see text) and the CD spectra scaled correspondingly. Arrows indicate regions in which the CD of Syn. 6803 appears composite with respect to the CD of spinach (see text).

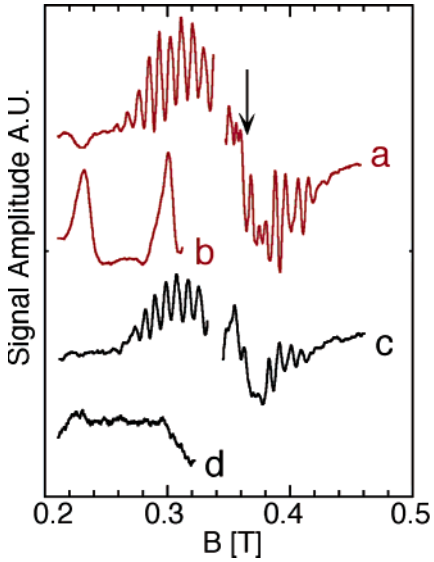

Figure 3. EPR spectra in the $g = 2$ region. (a) PSII cores illuminated at 245 K minus the dark spectrum, showing the S_2 multiline signal and the $Q_A^-Fe^{2+}$ signal (indicated with an arrow). (b) PSII cores dark spectrum showing the oxidized cyt b_{559} peaks. (c) BBY illuminated at 260 K minus the dark spectrum, showing the S_2 multiline signal and the $Q_A^-Fe^{2+}$ signal. (d) BBY dark spectrum. EPR settings: microwave frequency 9.42 GHz and power 5.0 mW, modulation frequency 100 kHz and amplitude 20 G, sample temperature 8 K.

sity distributions, makes any such analysis strongly undetermined. It is self-evident that the detailed structure and particularly the intensity distributions are significantly different between the two core complexes.

The cyanobacterial spectrum shows a strong negative CD peaking at ∼13 cm^{-1} lower energy (684.1 nm) than the corresponding CD feature in plants (683.5 nm). In addition to the peak at 684.1 nm, the cyanobacterial CD in this region has a shoulder near 682 nm, giving rise to a clearly composite negative CD feature of width 3−4 nm. This composite characteristic of the CD is in contrast to that of the plant CD feature, in which only a single feature is evident. Here, it has identical width (2 nm) and position to the 2.2-chl intensity absorption peak at 683.5 nm.

There are notable differences in both the CD and absorption spectra near 670 nm. In this region, the cyanobacterial system shows two distinct features and again the plant system only has one. Furthermore, the cyanobacterial system shows some structure on the blue slope, which is less evident in the plant system. The features of 2−3 nm width in the two spectra, whose peak positions were estimated from the second derivative of the absorption, are listed in Table 1 (Q_y section).

Spectral Changes of Spinach PSII Cores upon $S_1Q_A \rightarrow S_2Q_A^-$ Charge Separation. By illuminating our PSII core samples at 260 K with green light for 6 s, we create the $S_2Q_A^-$ charge-separated state. This state is trapped by immediately freezing the sample into He*(l)*. In Figure 3a, EPR data from a parallel sample are displayed, confirming that successful charge separation results from this protocol: a strong S_2 multiline signal has appeared, along with the spin-coupled $Fe^{2+}Q_A^-$ signal ($g = 1.98$, indicated with an arrow).

In Figure 4a, the pheo *a* Q_x absorption band is displayed for the PSII cores in the dark (red) and after illumination (blue). The band is composed of both D1- and D2-pheos. From a comparison of line shapes before and after illumination, it appears that the lower-energy component of the band is shifted to higher energy. The peak maximum shifts by 61 cm^{-1}, from 546.1 to 544.3 nm when Q_A is reduced. A blue shift at this wavelength has been noted previously, and the shift has been attributed to $Pheo_{D1}$ shifting electrochromically as a response to the negative charge on Q_A.

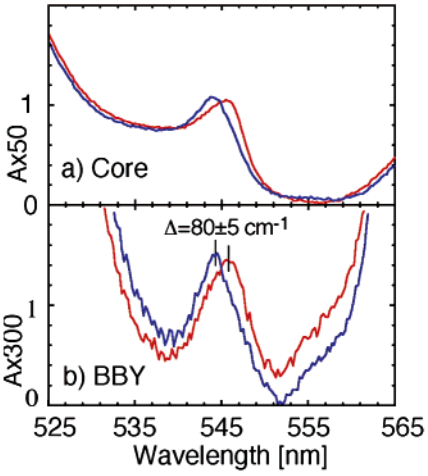

Figure 4. Pheo *a* Q_x region for (a) cores and (b) BBY, recorded prior to (red) and after (blue) illumination at 260 K. BBY data were recorded from DCMU-containing samples, and have had a linear baseline subtracted for clarity.

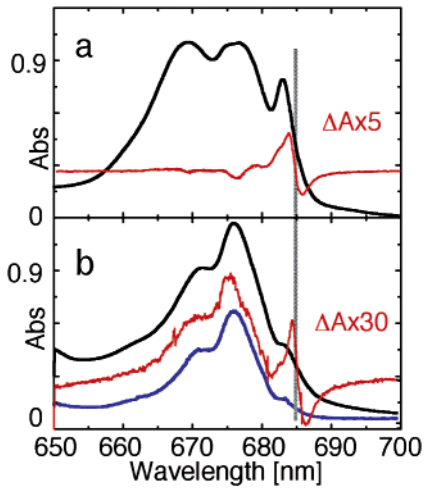

Figure 5. Q_y region for (a) cores and (b) BBY. 1.7-K spectra recorded prior to illumination are displayed (black), along with the absorption differences (ΔA) induced by 260-K illumination (red, data magnified as indicated). The blue line signifies the spectral change induced in BBY upon thawing to 260 K and reglassing in the dark.

No features are observed in the 555−560 nm region, where reduced cyt b_{559} absorbs, for the dark-adapted sample. This is in accordance with the EPR spectrum of the dark-adapted sample in Figure 3b, showing strong signals at $g = 3$ and $g = 2.27$ originating from oxidized cyt b_{559}. Surprisingly, a minor amount of cyt reduction is observed upon illumination (Figure 3a, 4a).

In Figure 5a we present the Q_y-region absorption spectrum of the PSII cores poised in the S_1Q_A state, along with the subtraction of this spectrum from that of the same sample after conversion to the $S_2Q_A^-$ state. Apart from a linear correction for a small change in sample scattering after reglassing, no further manipulations have been made to the data.

The main shift in this region is located (crosses zero) at 685.0 nm. As $Pheo_{D1}$ is the pigment in closest proximity to Q_A (12−13 Å),[3,4] and the $Pheo_{D1}$ Q_x transition clearly shifts upon Q_A reduction, we attribute the 685.0-nm shift to the $Pheo_{D1}$ Q_y transition. We note that the shift is asymmetrical, indicating that it may be of composite nature.

Spectral Changes of BBY Complexes upon $S_1Q_A \rightarrow S_2Q_A^-$ Charge Separation. With DCMU present to block electron transfer from Q_A to Q_B, PSII membrane fragments perform charge separation from the Mn cluster to Q_A upon 260-K illumination. Figure 3c displays the S_2 multiline EPR signal and the $Q_A^-Fe^{2+}$ indicative of successful charge separation in this material. In Figure 4b, the pheo Q_x region of the absorption spectrum is displayed before (red) and after illumination (blue). To make the pheo Q_x feature more easily visible, as it occurs as a relatively weak feature on a background absorption associated with the chl B-band in the BBY material, we have approximated the background absorption over this small spectral region to a straight line and subtracted this linear component from the raw data. As in the core results of Figure 4a, the dark spectrum displays a pheo peak of considerably lower energy than the illuminated spectrum. The shift of the peak maximum is (80 ± 5) cm^{-1} to the blue. At 556 nm, the absorption peak of reduced cyt b_{559} is present, at roughly equal amounts before and after illumination. This corroborates the EPR data of Figure 3c, showing that no redox chemistry involving the cytochrome is taking place during the illumination procedure. The amount of reduced cyt b_{559} present in the optical sample is less than expected from the EPR data, as Figure 3d shows very little oxidized cyt b_{559}. We frequently observe this discrepancy between our optical and EPR samples, although they are prepared very similarly.

Figure 5b shows the BBY absorption spectrum in the Q_y region (black), the changes induced by the 260-K illumination (red), and the changes induced by simply bringing the sample to 260 K in the dark and refreezing it (blue). There is a characteristic change in the absorption of the sample upon thawing and refreezing. This only occurs with BBY samples, and we ascribe it to changes in the glass properties, related to the large BBY particle size. However, there is a distinct feature induced by 260-K illumination which does not take place in the dark thaw/freeze process: a strong blue-shift crossing zero at 685.8 nm. This shift accompanies the pheo Q_x shift, and we ascribe it to the $Pheo_{D1}$ Q_y transition. It is similar, in energy, width and shape, to that observed in the core preparations (Figure 5a).

We estimate the peak molar absorption, ϵ_{max}, in the Q_y region to be $2.1-2.5 \times 10^6$ $M^{-1}cm^{-1}$ for our plant core complexes at 1.7 K. This value was estimated by a number of procedures. First, the oscillator strength of the Q_y absorption was equated to 33 times the oscillator strength of chl *a* in CCl_4. Second, the oscillator strength of β-carotene was compared to the area of the peaks in the 450−500-nm region, knowing our sample has 7 β-carotenes per reaction center. We also used the value of the oscillator strength of Q_x pheo *a* from Houssier and Sauer.[36] Finally, an estimate of the molar extinction of the core complexes was computed from the chl concentration (using 32 chl *a* per PSII) in our sample, dilution factors and path-length of the cell.

The corresponding ϵ_{max} value for BBY complexes is 6.0 ± 0.2 times that of cores ($(12.6-15) \times 10^6$ $M^{-1}cm^{-1}$). A subtraction of spectra before and after illumination of BBY material (the spectral data of Figure 4b) provides an estimate of the amplitude of the resulting $\Delta\epsilon_{550}$ feature of 30 000 $M^{-1}cm^{-1}$. In ref 31, very high Q_A^- yield was achieved in cyanobacterial PSII cores. Quantification of the data in Figure 1 of ref 31 using the same value of ϵ_{max} as for our plant core complexes renders a value of the analogous cyanobacterial $\Delta\epsilon_{550}$

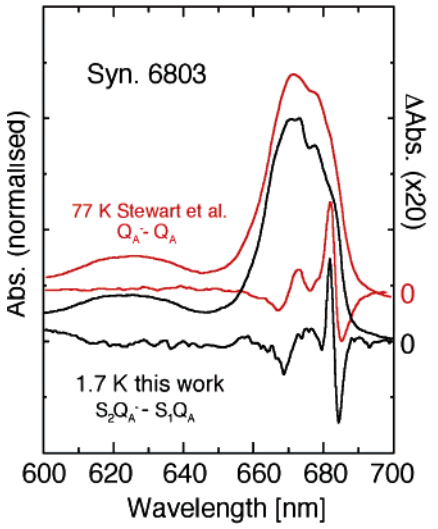

Figure 6. Black: absorption in the Q_y region of enzymatically active Syn. 6803 PSII complexes measured at 1.7 K and the change in absorption (ΔAbs) between spectra before and after illumination at 260 K. Red: 77-K data adapted from ref 31 for Mn-depleted PSII complexes prepared from the same organism, along with the corresponding change in absorption after illumination protocols as specified. The two absorption spectra have been scaled to the same area in the region presented and difference spectra adjusted accordingly. Spectra are offset in the diagram for clarity.

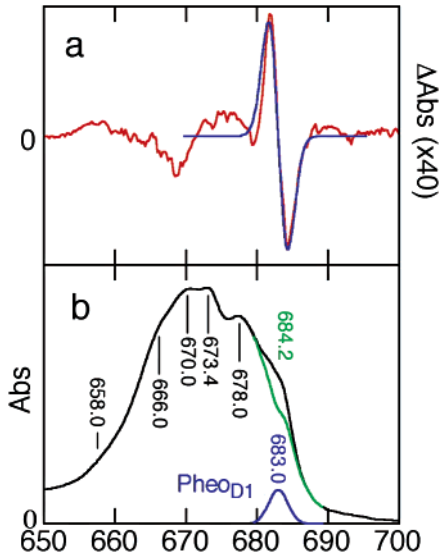

Figure 7. (a) Change in Syn. 6803 PSII Q_y absorption calculated from a subtraction of spectra taken before and after illumination at 260 K (from Figure 6), along with a fit of the electrochromism associated with $Pheo_{D1}$ (blue) generated by a 0.2 nm shift of the absorption shown in blue in (b) (see text). (b) The absorption spectrum of Syn. 6803 PSII at 1.7 K together with the band corresponding to the Q_y of $Pheo_{D1}$ (blue) (see text). The spectrum of the core complex with the $Pheo_{D1}$ band subtracted is also presented (green) along with wavelength positions of other resolved features in the spectrum.

feature of 23 000 $M^{-1}cm^{-1}$. After taking into consideration that our features are slightly narrower than those reported by Stewart et al.,[31] the $\Delta\epsilon_{550}$ appears to be of similar magnitude in the two experiments. This confirms the EPR data showing that in BBYs, 260-K illumination achieves close to stoichiometric creation of Q_A^-.

Spectral Changes of Syn. 6803 PSII upon $S_1Q_A \rightarrow S_2Q_A^-$ Charge Separation. In Figure 6 we present the Q_y-region absorption of dark-adapted cyanobacterial PSII complexes, along with the difference of the spectra obtained after and prior to 260-K illumination (black lines).

In this material, charge separation gives rise to a blue shift of a feature at 683.0 nm. Being the dominant feature of the absorption difference spectrum, we ascribe it to a shift of the cyanobacterial $Pheo_{D1}$. The shift is centered to the blue of the corresponding shift in plants, and also at slightly higher energy than the 684.1-nm CD feature identified in Figure 2. The shift is also notably more symmetrical than in the plant material.

The changes we see in the Q_y spectrum upon conversion of our Syn. 6803 sample to the $S_2Q_A^-$ state are compatible with shifts being small compared to the line widths of the spectral features (~3 nm or ~65 cm^{-1}). Starting within the approximation that the shift is much smaller than the line width of the $Pheo_{D1}$ absorption, integration of the difference spectrum (enlarged in Figure 7a) gives the position of the absorption to be 682.98 ± 0.02 nm with a fwhm of 3.2 ± 0.1 nm. The corresponding amplitude of the Q_y absorption of $Pheo_{D1}$ can be estimated from the pigment content of cyanobacterial core complexes (~32 chl *a* per core). If we use the solution value of the ratio of Q_y dipole strengths of pheo *a*/chl *a* of 0.7[36] and a total effective pigment content of 33 chl *a* per PSII complex (see above), then the contribution of $Pheo_{D1}$ to the Syn. 6803 core absorption (assuming a Gaussian line shape) is as shown in blue in Figure 7b. That is, the amplitude of the simulated $Pheo_{D1}$ feature has been adjusted to be 0.7/33 of the area of the entire band. The absorption envelope, with this feature subtracted, is indicated in green. This spectrum with the $Pheo_{D1}$ band removed shows a clear shoulder at 684.2 nm, approximately the same position as the sharp negative CD feature (Figure 2).

A blue shift of 0.2 ± 0.02 nm of the simulated $Pheo_{D1}$ feature reproduces the observed electrochromic shift with good precision (Figure 7a, blue line). This value of 0.2 nm is a lower limit for the blue shift, as several factors may lead to an underestimation of the shift: an incomplete $S_1Q_A \rightarrow S_2Q_A^-$ conversion, a reduction of the pheo *a*/chl *a* Q_y dipole strength ratio to a lower value than 0.7, or an increase in total pigment content of a PSII complex to >33 chl *a*. We note that the oxygen-evolving activity of our Syn. 6803 core material was not as high as for our plant core material. Taking these factors into account, the full $Pheo_{D1}$ Q_y shift resulting from Q_A reduction is probably 0.25−0.4 nm (5−9 cm^{-1}).

Also displayed in Figure 7b are other distinguishable features of the cyanobacterial PSII Q_y absorption envelope, which are incorporated in Table 1, along with corresponding features from PSII core complexes from spinach. Table 1 also specifies the Syn. 6803 pheo Q_x positions with Q_A neutral and reduced (spectra not shown). This band is 3.5 nm (120 cm^{-1}) to higher energy in the Syn. 6803 PSII compared with the spinach system, but undergoes a shift of similar magnitude upon Q_A reduction.

Spectral Changes of Spinach Cores and BBY upon 1.7-K Illumination. In the Materials and Methods section, it was noted that low-intensity illumination incident on a sample, even at the level present in a high-resolution scanning spectrometer, can give rise to significant spectral changes in enzymatically active PSII samples at low temperatures. We addressed this by reducing the light fluence at the sample to around 10^{10} photons/sec/cm^2 (detailed in Supporting Information), thus minimizing spectral changes while still providing the sensitivity needed to see small changes in absorption spectra.

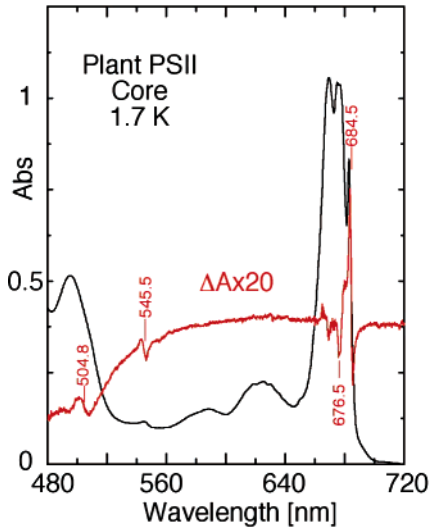

Figure 8. Visible spectral region for spinach PSII cores recorded at 1.7 K prior to illumination, displayed along with the absorption differences (ΔA) induced by 1.7-K illumination (data magnified as indicated).

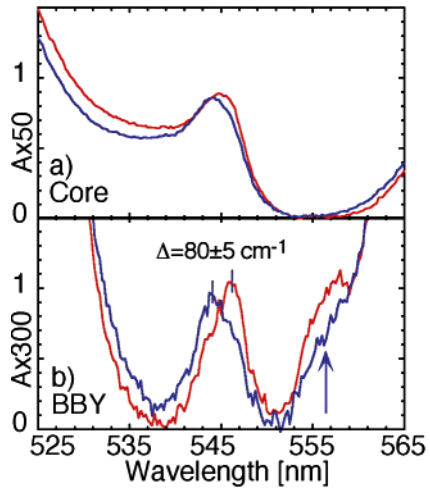

Figure 9. Pheo *a* Q_x region for (a) cores and (b) BBY, recorded prior to (red) and after (blue) illumination at 1.7 K. BBY data have had a linear baseline subtracted for clarity. The reduced cyt b_{559} absorption band at 556 nm is indicated.

To characterize the light-induced processes active at 1.7 K, we illuminated a core sample deliberately for 3 s at this temperature with ~2 mW/cm^2 of green light. This illumination corresponds to a total of $\sim 6 \times 10^{15}$ incident photons, of which an estimated ~10% would be absorbed by our samples (Q_y peak OD $\approx$ 1). Figure 8 shows a dark-adapted core spectrum (recorded with low-fluence light) and the difference induced by deliberate 1.7-K illumination, magnified 20 times. In Figure 9a, the spectra before and after illumination for the Q_x region are presented, whereas Figure 10a shows an expanded view of the difference in the Q_y region. Figures 9b and 10b show the equivalent data from BBY. Similar shift patterns were observed, preliminarily, from Syn. 6803 (not shown).

At this very low temperature few light-induced changes can be expected to occur in the sample, except for electron transfer. The data in Figures 8−10 clearly point to efficient formation of Q_A^- upon 1.7-K illumination. There is a characteristic large shift of $Pheo_{D1}$ Q_x, and a corresponding shift in the Q_y region, present in both BBY (685.9 nm) and core complexes (685.2 nm). These shifts are very similar in shape, width and energy

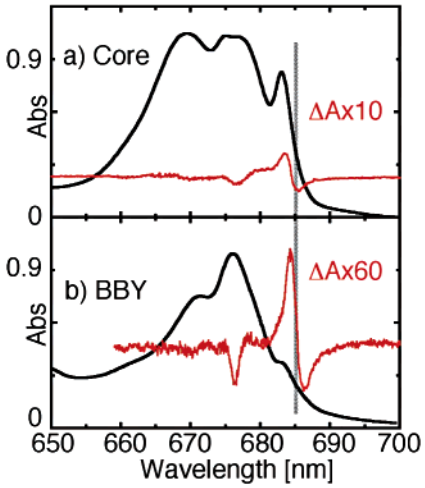

Figure 10. Q_y region for (a) cores and (b) BBY. 1.7-K spectra recorded prior to illumination are displayed (black), along with the absorption differences (ΔA) induced by 1.7-K illumination (red, data magnified as indicated).

to the $Pheo_{D1}$ Q_x and Q_y shifts identified after $S_1Q_A \rightarrow S_2Q_A^-$ turnover (Figures 4, 5). The amplitude of the Q_y shift (Figure 10) is approximately half that of the higher-temperature turnovers. By extending the illumination period to 30 s (not shown), the 685-nm shift increases to >90% of the high-temperature maximum, showing that complete Q_A reduction is easily achievable at 1.7 K.

The Q_y shift obtained from spinach PSII is invariably asymmetrical in the different preparations and at different illumination temperatures, indicating that this is an intrinsic characteristic of the system. A narrow (fwhm 1.5 nm, 50 cm^{-1}) feature is apparent at 676.9 nm in the BBY data (Figure 10b). A comparable negative feature is present in the core data at the same energy, but not as narrow. The induced spectral changes were stable at 1.7 K.

All of the pairs of dark and illuminated Q_x absorption spectra (Figures 4 and 9) are qualitatively and quantitatively quite similar. The clearest distinction between the dark and illuminated data is found in BBYs, particularly in the 1.7-K-illuminated samples (Figure 9b). From these traces, we determine the shift of the peak maximum to be (80 ± 5) cm^{-1}, from 546.2 nm in the dark to 543.9 nm after illumination and Q_A reduction. We note that the high-energy (blue) slope of the pheo feature is unchanged during the shift, and conclude that the shift of the peak maximum represents a lower limit of the shift of an underlying component, in this case $Pheo_{D1}$.

In Figure 9b, an absorption feature from reduced cyt b_{559} is present before the 1.7-K illumination, but diminished afterward, thus pinpointing the population of reduced cytochrome as an electron donor under these conditions. (This was confirmed by EPR, not shown.) A subsequent annealing of the 1.7-K-illuminated BBY sample at 250 K for 5 min relieves the electrochromic shifts of Pheo Q_y and Q_x, but the 556-nm band does not regain its initial intensity; the cyt b_{559} thus remains oxidized. A repetition of the low-temperature illumination after annealing leads to a regeneration of the electrochromic signals with high yield.

The value for $\Delta\epsilon$, the change in absorption from reduced to oxidized cyt b_{559} at the peak of the reduced absorption feature,

has been determined[38] as 21 500 $M^{-1}cm^{-1}$ from measurements on isolated cyt b_{559} preparations. The width of the difference feature in these studies is ~12 nm (at room temperature), whereas in Figure 9b it is around 4.8 nm (at 1.7 K). The latter width is in agreement with spectra taken on chemically reduced core complexes and D1/D2/b559 preparations at low temperatures.[39] Assuming that the oscillator strengths of the cyt b_{559} transitions in their oxidized and reduced forms do not vary in going from the isolated protein to the active PSII complex, we can correct the $\Delta\epsilon$ for the change in width. Using this and the BBY ϵ_{max} estimated above, ~25% of the cyt b_{559} population is in the reduced form prior to illumination.

In the cores, no reduced cyt b_{559} is present (Figure 9a). A significant decrease in absorption in the carotenoid region around 450−500 nm occurs upon illumination, in parallel with the observation of the growth of a band (spectra not shown) at 990 nm associated with carotenoid radicals.[14,15] This indicates that electrons are taken from β-carotene in the cores. Both the cores and BBY show overall conservative shift patterns in the chl Q_y region, signifying that very little chl (<0.1 chl per reaction center) has been oxidized.

The absorption difference in Figure 8 also reveals a differential-type feature centered at 504.8 nm, with $\Delta\epsilon = 8000$ $M^{-1}cm^{-1}$. A weak, relatively sharp shoulder near 505 nm can be discerned in the corresponding absorption spectrum. This feature may originate from one of the core carotenes, experiencing electrochromism upon 1.7-K illumination.

Quantum Efficiency of PSII at 1.7 K. As noted in Materials and Methods, PSII performed photodriven Q_A reduction at 1.7 K as a result of the spectrometer light incident on the sample when wide slits (500 μm) were used. Because the CD detection of our spectrometer is shot-noise limited, we can use the detected signal-to-noise ratio to estimate the number of photons incident on the sample per second. From the absorption spectrum we further estimate the fraction of these photons absorbed by our PSII material. Using this information, in conjunction with the number of PSII centers present in the interrogated sample volume and the amount of Q_A reduction induced, we estimated the ratio of Q_A reduction per photon absorbed (detailed in the Supporting Information). We find, that the quantum efficiency of Q_A reduction in PSII cores at 1.7 K is on the order of 0.1−1.

This estimation of the PSII quantum efficiency approaches the theoretical maximum, and substantiates the view of PSII as a highly efficient photoconverter. Tens of absorbed photons, or even less, are sufficient for Q_A reduction to take place in a single PSII center. This also puts some of the illumination protocols used on PSII material, such as white light for tens of minutes, into perspective.

Discussion

Pheophytin Q_x and Q_y Transitions in Spinach Preparations. The "C550 shift", arising from Stark shifts of the Q_x pheo band, is well-known and frequently detected by flash difference spectroscopy. We present precision absorption data taken before and after illumination. This approach allows the magnitudes of the Stark shifts to be accurately determined. This is especially so when the shifts are so large that they become comparable to spectral line widths. Using very low light levels in the spectrometer we have minimized measurement-induced changes in spectra.

In both cores and membrane fragments, before and after illumination, the Q_x band has an asymmetric character. In 5-chl D1/D2/b559 material,[39] hole-burning and chemical reduction studies showed $Pheo_{D1}$ absorbing at 544.4 nm and $Pheo_{D2}$ at 541.2 nm, approximately 130 cm^{-1} apart but spectrally overlapping. If the separation of the two pheos is similar in dark-adapted BBYs, then we can account for the observed asymmetric behavior. A Q_x Stark shift of $Pheo_{D1}$ to the blue of 80 cm^{-1} or more brings it closer to resonance with $Pheo_{D2}$. As $Pheo_{D2}$ is ~24 Å from Q_A, some Stark shift of $Pheo_{D2}$ Q_x cannot be excluded as a contribution to the overall line-shape changes observed. Given this complexity, along with the asymmetric Stark shift behavior in Q_y, we do not have sufficient data at present to further quantify this process. We are, however, able to establish a lower limit of 80 cm^{-1} for the $Pheo_{D1}$ Stark shift as a result of Q_A reduction from the shift of the peak maxima.

This appears to be the first quantitative determination reported for the Q_A-induced $Pheo_{D1}$ Q_x Stark shift for sub-chloroplast preparations. (The equivalent shift was measured in whole chloroplasts[40] and quantified to 55 cm^{-1} in the thesis of Hans van Gorkom, personal communication.) Following Mulkidjanian et al.,[30] we can use the now known distance between $Pheo_{D1}$ and Q_A of 12 Å[3] and our observed shift of 80 cm^{-1} to establish a minimum value for $\Delta\mu_x$, the change in dipole moment between ground and excited state for the Q_x transition. Using a dielectric constant of $\epsilon_{eff} = 1-2$ (appropriate for the protein interior at cryogenic temperatures[41]) we obtain $\Delta\mu_x \geq 0.5$ D. This value depends on the angle between $\Delta\mu_x$ and the $Pheo_{D1}-Q_A$ distance vector; the lower limit applies if these are parallel.

The Q_y shift for $Pheo_{D1}$ in spinach cores is similarly obtained, and close in magnitude to that estimated above for Syn. 6803 PSII cores, 5−9 cm^{-1}. This is an order of magnitude smaller than the value observed for the corresponding Q_x transition. Assuming that $\Delta\mu_x$ and $\Delta\mu_y$ are approximately perpendicular to one another and of comparable magnitude, this ratio of 10:1 of the Q_x and Q_y shifts puts constraints on the direction of $\Delta\mu_y$ in $Pheo_{D1}$. Only if $\Delta\mu_y$ is near perpendicular to the $Pheo_{D1}$-Q_A distance vector (5 degrees in either direction) can the observed shifts be accounted for, assuming reasonable limits for the $\Delta\mu$ magnitudes. Figure 11 shows $Pheo_{D1}$ and Q_A, deduced from ref 3, where the position of ring V of the $Pheo_{D1}$ chlorin was established for cyanobacterial PSII. This assignment is in agreement with that of the homologous bacterial reaction center, which has been structurally determined to higher resolution. The direction of $\Delta\mu_y$ consistent with our observations is displayed, along with the corresponding $\Delta\mu_x$, placed perpendicular to $\Delta\mu_y$. The direction of the observed shifts (toward higher energy upon Q_A reduction) dictates that both $\Delta\mu_x$ and $\Delta\mu_y$ point away from Q_A. This assignment of the directions of change of dipole moments indicates that the size of $\Delta\mu_x$ is in fact near its lower limit of 0.5 D, discussed previously.

The photodriven reduction of Q_A has been established by EPR spectroscopy.[28,29] As the EPR signal arising from this species (spin-coupled to the non-heme Fe^{II}) is identical after illumination

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(39) Jankowiak, R.; Rätsep, M.; Picorel, R.; Seibert, M.; Small, G. J. *J. Phys. Chem. B* **1999**, *103*, 9759−9767.
(40) Butler, W. L.; Okayama, S. *Biochim. Biophys. Acta* **1971**, *245*, 237−239.
(41) Steffen, M. A.; Lao, K.; Boxer, S. G. *Science* **1994**, *264*, 810−816.

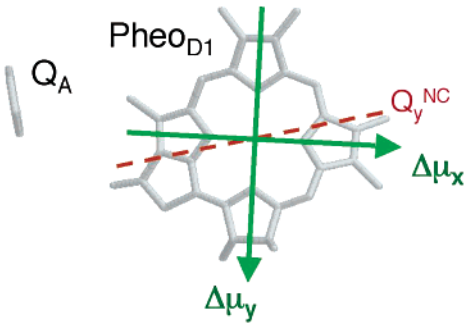

Figure 11. Pheo$_{D1}$ and Q_A, adapted from Kamiya and Shen.[3] The direction of $\Delta\mu_y$ deduced from our data is shown (95 degrees from the Q_A-Pheo$_{D1}$ center-to-center vector), as well as the corresponding $\Delta\mu_x$ if these are assumed to be perpendicular. The direction of the Q_y transition moment measured for pheo *a* in nitrocellulose (NC)[43] is also indicated.

at 5 K[28] and at 200 K, it appears that Q_A reduction is a pure electron-transfer event and not associated with proton rearrangements that may move the effective center of charge away from the quinone. Thus, our assumption that the Pheo$_{D1}$-Q_A distance vector represents the field direction when determining the $\Delta\mu$ directions is justified.

For chl *a* in solution, the direction of $\Delta\mu_y$ lies within 20° of the Q_y transition dipole.[42] The Q_y transition dipole has been measured for pheo *a* oriented in a nitrocellulose matrix,[43] the result of which is displayed in Figure 11 (marked Q_y^{NC}). LD measurements conducted by Germano et al. on D1/D2/b559 particles whose pheos were substituted by chemically modified pheos, indicated that the Q_x transition of both pheos were polarized parallel to the membrane plane and the Q_y transitions perpendicular to that plane.[37] Considering the positioning of Pheo$_{D1}$ with respect to the membrane plane, these findings are consistent with the nitrocellulose result. Comparing these data to our present observations, it is unlikely that the transition dipoles and the $\Delta\mu$ vectors of Pheo$_{D1}$ are close to parallel, as they are in chl *a*. This may be evidence of protein-induced changes in the electronic properties of pigments, specifically the photochemically active Pheo$_{D1}$.

In D1/D2/b559 particles, the pheo Q_x band is centered at 543.5 nm for 6-chl preparations[37] and 542.8 nm for 5-chl preparations.[39] Comparing these values to the core and BBY data (with Q_A neutral, because Q_A is absent in these PSII centers), this shows a significant shift to higher energy of the pheos upon removal of the inner antenna. Interestingly, the pheo Q_x transition energy in plant-derived D1/D2/b559 material is closer to that of Syn. 6803 cores (see Table 1).

Pheophytin Q_y transition in Synechocystis. In Figure 6, our absorption and $S_2Q_A^- - S_1Q_A$ difference spectra from Syn. 6803 PSII are compared to a 77-K spectrum of Mn-depleted Syn. 6803 core complexes treated with $K_3Fe(CN)_6$, as well as the corresponding illumination-induced $Q_A^- - Q_A$ difference spectrum. These latter data were digitized from the curves shown in Figure 1 of ref 31. The detailed protocol established in the latter work was shown to create a high yield of Q_A^- after illumination, with minimal pigment or tyrosine oxidation. The chemical reductant used was hydroxylamine, which was in excess in the sample.

The correspondence in Figure 6 between our difference data and those from Mn-depleted material is remarkably close. Our (lower-temperature) absorption spectrum shows significantly finer detail and our difference spectra, although again better resolved, have somewhat poorer signal-to-noise. This is due, in part, to the need to use low light levels in absorption measurements (see Materials and Methods). Our data present the absorption and uncorrected (except as noted above) difference spectra, whereas the data from Stewart et al.[31] contain an unspecified correction to the total area of the shift so as to maintain constant Q_y absorption area in the spectra. This inevitably leads to a purely conservative difference spectrum in the Q_y region but we note that it leads to an absorption loss in the 560−570 nm region that is not accounted for. Overall, the changes we see in the Q_x region of Pheo$_{D1}$ (Table 1) are also similar to those seen by Stewart et al.[31]

The comparison in Figure 6 establishes that the Q_A reduction protocol used by Stewart et al.[31] and the illumination procedure used here result in equivalent states. It also shows that the presence of the Mn cluster has no observable effect on the measurement, and that the S_1-to-S_2 transition of the Mn cluster does not create an observable Stark shift on the core pigments. Similarly, the shift patterns observed from our spinach PSII cores upon illumination at 260 K (causing Mn oxidation) and at 1.7 K (where an alternative electron donor is active) are very similar, indicating that Mn oxidation causes minor or no electrochromic effects on the spinach core pigments. This can be compared to a significant electrochromic shift of the 683.5-nm feature observed upon conversion of the Mn cluster from the "multiline" to the "$g = 4.1$" form of the S_2 state.[8] These results differ from room-temperature flash difference absorption data from pea PSII cores,[30] spinach BBY,[44] and Synechococcus cores,[45] which all report transient blue shifts in the 680-nm region associated with the S_1-to-S_2 transition of the Mn cluster. This apparent discrepancy may be explained if the initial Mn oxidation, detected 30 ms after the flash in the kinetic experiments,[30] is followed by slower proton rearrangements that compensate for the charge build-up. These would have proceeded to completion on the time scale of our 260-K illumination.

The magnitude of the electrochromic shift of the Q_y band of Pheo$_{D1}$ determined above (a 0.25−0.4-nm shift centered at 685.0 nm) is in approximate agreement with the analysis of room-temperature transient-absorption changes in pea PSII.[30] In that material, the Q_y band shift associated with Q_A^- formation was reported to be 0.223 nm of a band at 684.6 nm with a width of 11.7 nm. However, as the local dielectric in a reaction center has been determined to be twice as large at room temperature as at 77 K and below,[41] the shift on Pheo$_{D1}$ is expected to be twice as large at low temperatures. The fact that our shift is less than twice that of ref 30 may in part be due to partial Q_A^- decay during our freezing procedure, an effect that would not affect transient measurements.

The corresponding 77-K-band shifts reported by Stewart et al.[31] on Mn-depleted Syn. 6803 are, however, almost a factor of 20 greater (3.9 ± 0.2 nm) than those reported here and in ref 30. To obtain these large shifts, a far lower amplitude of the Pheo$_{D1}$ feature in the absorption has been invoked. From

(42) Krawczyk, S. *Biochim. Biophys. Acta* **1994**, *1056*, 64−70.
(43) van Zandvoort, M. A. M. J.; Wrobel, D.; Lettinga, P.; van Ginkel, G.; Levine, Y. K. *Photochem. Photobiol.* **1995**, *62*, 299−308.
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the lower panel of Figure 6 of this paper,[31] the effective dipole strength used by these workers can be determined. It is only 5% of the dipole strength of pheo *a* in solution. This value seems unacceptably low and suggests that the large inferred shift is a consequence of an inappropriately small value for the $Pheo_{D1}$ oscillator strength.

1.7-K Charge Separation. The strong $Pheo_{D1}$ shift created by illumination at 1.7 K clearly indicates efficient reduction of Q_A at low temperatures. The oxygen-evolving Mn cluster is not available as an electron donor at temperatures lower than 130 K. Alternative electron pathways on the PSII donor side have been explored in detail by others.[15,32] Our preliminary findings are in general agreement with their results: in BBYs we detect some cyt b_{559} oxidation accompanying Q_A reduction (Figure 8b), and in cores (where cyt b_{559} is oxidized) our results indicate carotenoid oxidation upon 1.7-K illumination (Figure 8). No observable chlorophyll oxidation takes place in our core or BBY samples upon 1.7-K illumination. A quantitative analysis of the electron pathway active under these conditions, including spectra from the radical-cation region of chlorophyll and β-carotene, will be presented separately.

The shifts obtained by 1.7-K illumination give us a view of the effect of Q_A reduction on the core pigments when all processes except electron transfer are inhibited. The data on BBY (Figure 10b) are particularly well resolved and informative. The shift of $Pheo_{D1}$ shows the same distinctive asymmetry as after higher temperature illuminations, indicating that this is intrinsic to this shift. Furthermore, the feature has a significant nonzero integral. There is also a remarkably sharp negative feature at 676.9 nm. The total integral over the Q_y shift spectrum is close to zero with the positive residual in the $Pheo_{D1}$ feature being accounted for by the sharp negative feature.

Depletion of a specific pigment near 676.9 nm seems an unlikely cause for this feature, as no equivalently narrow feature is discernible in the absorption spectrum. Any feature with an intensity close to one chl or one pheo having such a narrow spectral width would be easily discernible. It is more likely that the source of this behavior lies in a more involved rearrangement of chromophore intensities associated with electrochromic effects. An equivalent pattern is present in the core shift data, both after 1.7-K illumination (Figure 10a) and 260-K illumination (Figure 5a). We also note that in D1/D2/b559 material, some groups have associated a feature near 677 nm with the inactive pheo.[37,46] However, as the pheo Q_x band is significantly different in this material compared to more intact PSII (see above) the Q_y positions may also differ.

Spinach PSII Membrane Fragments and Core Complexes. Our spinach PSII cores are, uniquely, equivalent to the parent BBY material in terms of oxygen evolution rate per PSII.[7] As clearly seen in Figure 1, the sharp absorption/CD feature at 683.5 nm is also retained quantitatively from BBY to cores. The current report also clearly demonstrates that the positions of the $Pheo_{D1}$ Q_x and Q_y absorption bands are very similar in the two types of preparations, and that they shift similarly upon Q_A reduction. Taken together, this portrays the cores as a good model system, representative of intact PSII in terms of activity and spectral characteristics. The spectral detail of this preparation indicates high homogeneity of the material, and renders it suitable for more specific optical studies.

As mentioned previously, we have proposed[7,8] that the prominent 683.5-nm feature in plant PSII is associated with P680. The isolated CP43 material shows a distinct feature near 682 nm which has been studied by several groups[19−27] using techniques including hole burning and fluorescence line narrowing. In the terminology of ref 21, the feature is composed of two independent, quasi-degenerate Q_y trap states. The analysis in that paper identifies an "A" state with a width of 120 cm^{-1} at 683.3 nm and a narrower "B" state of width 45 cm^{-1} at 682.9 nm. If the 683.5-nm feature in assembled cores was related to these CP43 pigments, then this would account for the complete absence of such a feature in D1/D2/b559 particles. However, as indicated in ref 8, the absorption strength attributable to the sharp spectral features in CP43 *cannot* account for the intensity of the 683.5-nm absorption feature in plant core complexes. Neither can it account for the amplitude of its CD. The CD amplitude of CP43 is only comparable to that of the shoulder feature in the CD of Syn. 6803 near 682 nm in Figure 2 (see below). We will report such comparisons in more detail in a future publication.

PSII from Spinach and Synechocystis. The comparison of PSII cores from spinach and Syn. 6803 shows a similar overall Q_y envelope, with comparably well-resolved features having line widths of around 50−70 cm^{-1}. There are, however, very significant differences of the detailed spectral pattern, both in the absorption and the CD spectra (Figure 2).

As detailed in Table 1, the cyanobacterial PSII is well structured and shows a greater number of features than the plant material. There is no *single* feature of comparable intensity to the 2.2-chl feature in plants at 683.5 nm. There is a feature with strong negative CD at 684.1 nm in Syn. 6803 complexes. Tentatively, we associate it with the corresponding 683.5-nm feature in plants. The CD in cyanobacteria is composite (Figure 2) and exhibits a shoulder near 682 nm (see arrows). The magnitude of both the CD and the absorption strength from which it is derived is at least compatible with the 682-nm feature being associated with CP43. By inference, it seems possible that in the plant material, the narrow CP43 and more dominant P680 features are quasi degenerate. This degeneracy is removed in cyanobacteria, leading to a splitting in the CD in both the 683-nm region and the higher-energy 670-nm region. These components are marked with arrows in Figure 2.

Most studies of CP43 are performed on preparations extracted from PSII of plant origin. However at least one study of CP43 from a cyanobacterium (elongatus)[47] has been reported. The low-temperature spectra presented show little difference to spectra of CP43 obtained from plants, indicating that spectral changes of sharp features in core spectra sourced from plants and cyanobacteria may not arise from changes in the CP43 protein between different species. Overall, spectra of CP47 in plants are more weakly structured,[19,20,23−27] especially in the 683-nm region and again may not contribute significantly to the changes seen between core complexes prepared from different organisms. Therefore, the inference remains that the interspecies changes seen are mostly associated with changes in the photoactive pigments in the D1/D2 proteins. We currently have underway a detailed comparative study of CP43, CP47, D1/D2/b559

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fragments, combining absorption, CD, magnetic CD and hole-burning techniques, so as to further identify the spectral changes induced when an active core complex is disassembled into its constituent pigment−protein components.

The electrochromic effects associated with Q_A reduction are most evident in the Q_x region of the pheophytins. In the Q_y spectral region, parallel electrochromic effects are dominated by the shift of a well-defined spectral feature at 683 nm in Syn. 6803 and 685 nm in spinach. Both the Q_y and Pheo Q_x electrochromic effects occur at *higher* energy in Syn. 6803 compared to spinach. In the X-ray crystal structure of Kamiya and Shen[3] Q_A is far closer to $Pheo_{D1}$ (∼12 Å) than $Pheo_{D2}$ (∼24 Å). Q_A and $Pheo_{D1}$ are also within the same (D1) protein. Thus, far larger electrochromic shifts associated with the formation of Q_A^- are expected for $Pheo_{D1}$.

PSII from Cores to D1/D2/b559. A number of recent experiments[18,37,48] on D1/D2/b559 reaction-center preparations have led workers to conclude that $Pheo_{D1}$ is significantly coupled to other P680 pigments, and that $Pheo_{D1}$ absorbs at significantly higher energies than 685 nm. In particular, the pentamer model[18] has the $Pheo_{D1}$ absorption intensity distributed over a number of exciton bands between 670 and 680 nm. P680 models that place $Pheo_{D1}$ at higher energies,[39,46] or have the $Pheo_{D1}$ excitation substantially distributed over a number of exciton bands are not supported by our results. These models would either lead to a strong electrochromic shift observable at correspondingly higher energies or electrochromic effects distributed throughout the P680 region, reflecting the $Pheo_{D1}$ fractional occupation of that excitation, respectively. Our results would allow for a degree of delocalization of $Pheo_{D1}$ excitation (∼10−20%), but the dominant excitation is constrained to be located where the electrochromic effect is seen.

We have previously attributed the 683.5-nm feature in active spinach core complexes to P680.[7,8] Here, we suggest that, in Syn. 6803, the corresponding feature is at 684.1 nm, possibly having lower absorption strength. Between these two photosystem types, the relative energies and intensities of P680 chl pigments and $Pheo_{D1}$ have changed, yet the electrochromic signal is qualitatively and quantitatively similar. If $Pheo_{D1}$ were strongly coupled to P680, such an invariance of the electrochromism seems unlikely. We feel this provides further evidence for minimal coupling between $Pheo_{D1}$ and P680 in functional, intact PSII. We also feel that it indicates a significant variability of active P680 systems in nature. When the integral CP43 and CP47 proteins are removed, this inherent variability may lead to the collapse of structure in D1/D2/cytb559 preparations, leading to their strongly inhomogeneous behavior.

The coupling between pigments within the dipole−dipole (exciton) approximation, is governed by the inter-pigment distances and the relative directions of the transition dipoles, along with their magnitudes. Factors that immediately constrain the interaction between $Pheo_{D1}$ and the closest chl is the separation (12.4 Å) and the smaller transition dipole strength (×0.3−0.7) of $Pheo_{D1}$ compared to Chl. Recent crystallographic data has identified the 5th ring of the chlorins, but the precise direction of the transition dipole (Figure 11) is still subject to significant uncertainty,[43,49] especially considering that individual pigment properties may be significantly modified by the protein environment. The Q_y transition moment of $Pheo_{D1}$ is oriented nominally out of the membrane plane whereas the closest chl is nominally polarized in plane.[37] Thus, it is at least feasible that interactions could be minimal.

Our assignment of the unusual orientation of the $Pheo_{D1}$ $\Delta\mu$'s is dependent on the assumption of "normal" magnitudes of $\Delta\mu$'s. It is possible that the special pheophytin, which is the primary acceptor in PSII, has its $\Delta\mu$ values modified by interactions with the protein. We have shown that $\Delta\mu_x$ cannot be less than ∼0.5 D. If $\Delta\mu_x$ was much larger, and the value for $\Delta\mu_y$ also varied, a different orientational assignment would ensue. In either case, $Pheo_{D1}$ would have atypical properties.

Finally, there may be no need for the energy of $Pheo_{D1}$ to be closely resonant with P680 chl's. We note a recent report of a fully functional genetically modified Syn. 6803 mutant in which the active pheo *a* of PSII has been replaced with pheo *b* (Vavilin, Xu, Lin, and Vermaas, in press in *Biochemistry*).

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Supporting Information Available: Details on the calculation of the quantum efficiency of PSII. This material is available free of charge via the Internet at http://pubs.acs.org.

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