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Time-resolved spectroscopy at 10 K of the Photosystem II reaction center; deconvolution of the red absorption band

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The red absorption band of isolated Photosystem II reaction centers at 10 K and its flash-induced changes due to formation of the primary radical pair, P^+I^-, and subsequent recombination to the triplet state, P^T, were analysed. It is concluded that the primary electron donor P consists of two chlorophyll molecules, which show exciton interaction and are responsible both for the main long wavelength absorption band at 679.6 nm and a shoulder at 683.6 nm (these wavelengths vary somewhat between preparations). The angle between their Q_y transitions is about 60° and the exciton splitting about 85 cm^{-1}. Both bands are bleached in the states P^+ and P^T and replaced by a single band at 678 nm, attributed to one of the two chlorophylls, while the oxidized or triplet state is localized on the other, contributing little absorbance in the Q_y region. The pheophytin which acts as the intermediary electron acceptor I has a 6.4 nm wide Q_y band at 676.5 nm with about 2/3 of the amplitude of a chlorophyll Q_y band. For the remaining pigments gaussian curve fitting of the absorption spectrum led to the following tentative assignments. The Q_y band of the other pheophytin is spectrally indistinguishable from that of I. All accessory chlorophylls absorb in the 670 nm region; a good fit was obtained with two 4.7 nm wide bands peaking at 672.6 and 669.4 nm, respectively, and at least one Q_y band at slightly shorter wavelength. However, neither an artifactual origin of the latter band nor the presence of two such bands can be excluded. The pigments of the PS II reaction center show little exciton interaction.

Introduction

Optical spectroscopy of Photosystem II (PS II) reaction-center pigments is complicated by the fact that their absorption spectra are similar to those of the bulk antenna pigments. The isolation of pure reaction-centers, also called D_1D_2 cytochrome *b*-559 complex, by Nanba and Satoh [1] has not made it much easier. At the red end of the absorption spectrum of such a preparation all chlorophylls and pheophytins contribute to a single band around 675 nm. A selective bleaching on the long-wavelength side, around 680 nm, is observed upon oxidation of the primary electron donor P [2], reduction of the primary acceptor I [1], triplet formation [3] or inactivation [4]. To explain this uninformative spectroscopic behavior it has been proposed that the absorption properties are governed by excitonic interactions between all constituent pigments [5].

At present it is not even clear how many pigments there are. Indications for the presence of 4 chlorophyll *a* and 2 pheophytin *a* [6–8], which would be analogous to the pigments in reaction-centers of purple bacteria [9,10], suggest that preparations containing more chlorophylls may be less pure. However, recent spectroscopic evidence [11] and notably a careful quantitative HPLC analysis [12] indicated the presence of six (or even more) chlorophylls per two pheophytins,. and in fact there is also no compelling evidence for the generally assumed presence of two pheophytins in the isolated PS II reaction-center.

It is now clear that the presence of Triton X-100, the detergent generally used for the isolation of PS II reaction-centers, was responsible for some of the instability and low spectral resolution. Replacement by other detergents has led to more stable preparations [4] which at low temperature show some structure in the red absorption band [5]. We have used such a preparation to study the composition of the red absorption band at low temperature by time-resolved spectroscopy.

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Materials and Methods

The D_1D_2 cytochrome *b*-559 complex was isolated from spinach PS II membranes prepared according to Berthold et al. [13], using essentially the same procedure as Chapman et al. [14]. After dissociation of the extrinsic proteins in 50 mM Tris-HCl (pH 9.0) the PS II membranes were spun down at $40\,000 \times g$ and resuspended in 50 mM Tris-HCl, (pH 7.2). Triton X-100 was added to a final concentration of 4.25%. After 1 h incubation the suspension was centrifuged for 1 h at $100\,000 \times g$. The supernatant was loaded on a Fractogel TSK DEAE-650 column (Merck) and separated by washing extensively with 50 mM Tris-HCl, (pH 7.2), containing 0.2% Triton X-100 and 40 mM NaCl. The D_1D_2 cytochrome *b*-559 complex was eluted with a 40–200 mM NaCl gradient. To remove Triton X-100 the preparation was diluted and loaded on the same column, washed with buffer containing 40 mM NaCl and 2 mM dodecyl β,D-maltoside, and eluted again with an NaCl gradient. All procedures were carried out at 4° C and unnecessary illumination of the preparation was avoided. The final preparation had an absorption maximum at 675 nm. Samples were stored in the dark in liquid nitrogen. Before measurements a sample was thawed, diluted with two parts of glycerol and cooled to 6–15 K in a liquid-helium flow cryostat.

Ground state absorption spectra were measured with a single beam apparatus [15] at a spectral resolution of 0.65 nm.

Flash-induced absorbance differences on a picosecond time scale were measured with a pump-probe method using a passively mode-locked Nd : YAG laser. Frequency-doubled pulses of 532 nm, 25 ps FWHM duration and energy up to 10 mJ, were used for excitation. The remaining 1064 nm output of the laser was focused in a H_2O/D_2O mixture to generate a continuum probe pulse of about the same duration as the excitation pulse. The resulting instrument response function was a gaussian of 35 ps FWHM. The detector was an 1024-element diode array, EG&G model 1412, controlled by a Multichannel Analyser Controller, model 1218. One half of the array was used to measure the spectrum of the incident probe pulse, the other that of the transmitted probe pulse. The dispersion was 0.2 nm/channel but the spectral resolution was about 1.5 nm. 50–100 shots were averaged for each spectrum.

Flash-induced absorbance changes in the nanosecond to millisecond time range were measured as described by Smit et al. [16], except that for the nanosecond time range the measuring light was provided by a 15 μs xenon flash and for detection a fast photomultiplier and a home-built oscilloscope digitizing apparatus were used, with a response time of 1 ns. The spectral resolution was about 2 nm. The 15 ns FWHM, 532 nm flashes from a Q-switched frequency-doubled Nd:YAG laser were used for excitation. 60 shots were averaged for each kinetic trace. From measurements in the red region a flash artifact was subtracted.

Results

Absorption spectrum

The absorption spectra at 230 K and at 10 K of the Photosystem II reaction-center preparation are shown in Fig. 1A. The 230 K spectrum (dashed line) is similar to reported absorption spectra measured at 0° C or room temperature. The narrowing of absorption bands at 10 K clearly enhances the spectral resolution. Only the Soret region, below 450 nm, is remarkably little affected by the low temperature. Pronounced changes are observed in the carotene absorption bands, in the 450 to 510 nm range, and the pheophytin Q_x band at 542 nm is sharpened. The broad, flat-topped absorption around 675 nm is split into two main bands peaking at 671 and 679 nm and reveals some additional structure upon closer inspection, as illustrated by the second derivative spectrum in Fig. 1B. On the long-wavelength side the main 679 nm band has a shoulder around 683 nm, which results in a peak at 684.5 nm in the second derivative spectrum. The 671 nm band appears to consist of at least two unresolved bands; the second derivative shows peaks at 669 and 672 nm. To see if these

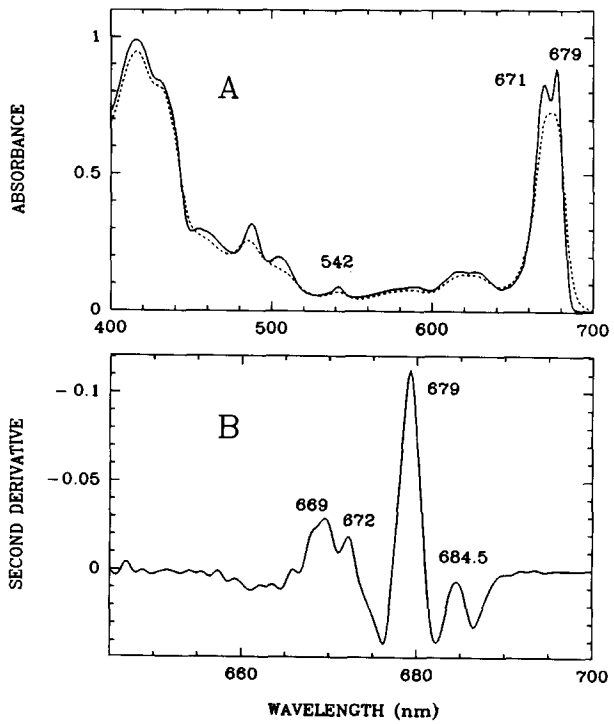

Fig. 1. (A) Absorption spectra at 10 K and 230 K (dashed line) of the D_1D_2 cytochrome *b*-559 complex isolated with the detergent Triton X-100, subsequently exchanged for dodecyl maltoside. (B) Second derivative of the absorption spectrum at 10 K in the red region.

bands result from complex exciton interactions or can be assigned to Q_y transitions of individual reaction-center pigments we have studied the light-induced changes of the absorbance in the Q_y region in detail.

Charge separation

In the picosecond time range the flash-induced difference spectrum in the Q_y absorption region was measured as a function of the delay time between the exciting and the measuring flash. At a delay of −20 ps (both pulses have a halfwidth of about 25 ps) a bleaching already develops both around 670 nm and around 680 nm (not shown). At a delay of 10 ps these have reached their maximum amplitude and a large fraction of the absorbance has disappeared (Fig. 2, trace 1). The absorbance around 670 nm partially recovers with a time constant of about 40 ps, close to the instrument response time (Fig. 3A). The remaining part of the bleaching around 670 nm, and most of that around 680 nm does not seem to decay on this time-scale. The maximum of the bleaching is shifted to longer wavelength (Fig. 2, curve 2). The absorbance changes at 10 ps may be largely due to excited states, whereas the long-lived absorbance changes presumably reflect the formation of the radical pair P^+I^-.

Charge recombination

The kinetics on a longer time scale, measured with a different apparatus, are illustrated in Fig. 3B and C. Both at 448 nm, near the 450 nm maximum in the $I^- - I$ difference spectrum [17], and at 674 nm a single exponential decay with a time constant of 100 ns is observed (Fig. 3B), while the bleaching at 680 nm is diminished by less than 20% in that time and largely decays with a time constant of 1.7 ms instead (Fig. 3C). A 100 ns recombination time for P^+I^- at 10 K seems reasonable in view of reported ($1/e$) times of 77 ns at 5

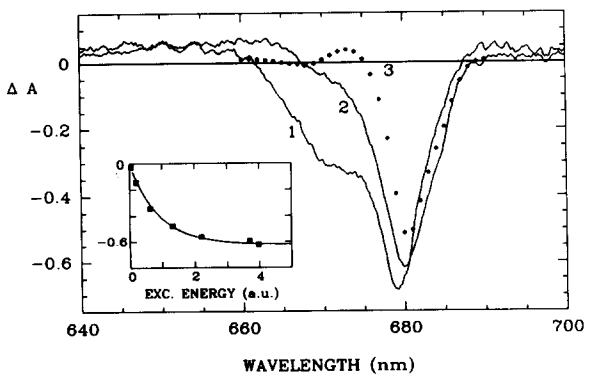

Fig. 2. Flash-induced absorbance difference spectra at 10 ps (curve 1) and 1.0 ns (curve 2) delay between actinic and measuring flash, and spectrum of flash-induced absorbance changes with a lifetime of 1.7 ms (curve 3). The inset shows the dependence on flash energy of the change at 680 nm at 1.0 ns. The absorbance of the sample at 679 nm was 1.35.

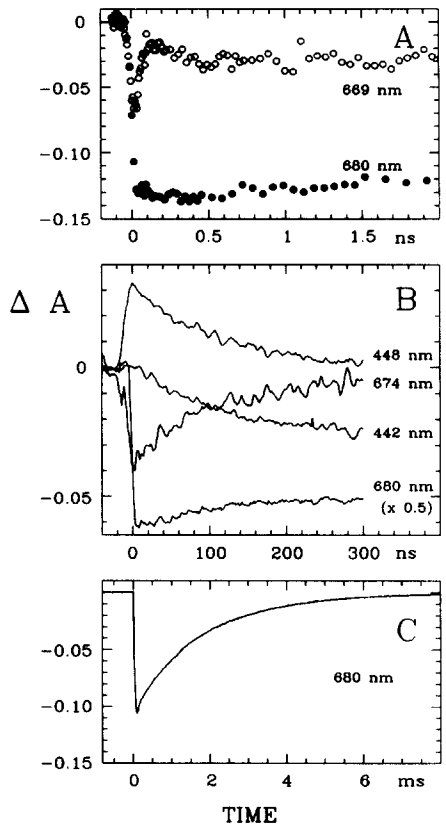

Fig. 3. Kinetics of flash-induced absorbance changes at the wavelengths indicated. Note the different time-scales.

K [3], 130 ns at 77 K [18], 60 ns at 120 K [3] and 50 ns at 278 K [19]. The absorbance changes with a lifetime of 1.7 ms are probably due to the triplet state: a 1.4 ms lifetime for the triplet state P^T, formed with an 80% flash yield at 5 K, has been reported before [3]. The spectrum of absorbance changes with a lifetime of 1.7 ms (Fig. 2, curve 3) consists mainly of an asymmetrical, 6–7 nm wide band peaking near 680.5 nm. Its precise shape on the short-wavelength side varied somewhat between preparations and we have the impression that prolonged storage may lead to a wider, more symmetrical bandshape similar to the triplet minus singlet difference spectrum measured by ADMR at 1.2 K in a *Chlamydomonas* PS II core particle [20]. We conclude that the radical pair P^+I^- decays in 100 ns, largely to the triplet state P^T which decays in 1.7 ms to the ground state. Since the difference spectra of $P^+ - P$ and $P^T - P$ may be similar in the Q_y region, the additional bleaching around 675 nm in the P^+I^- spectrum, which is reversed in 100 ns (Fig. 3B), is tentatively attributed to the Q_y absorption band of the primary acceptor I. This assignment is consistent with the linear dichroism changes observed upon photoaccumulation of I^- [21].

Special attention should be paid to the amplitude of the absorbance changes. At saturating flash energy (Fig. 2, inset) the absorbance at 685 nm disappears almost

completely upon formation of P^+I^- and that at 680 nm is about halved.

Discussion

We have shown that the Photosystem II reaction-center preparation, isolated with Triton X- 100 followed by exchange of this detergent for dodecyl maltoside, displays a partially resolved structure of the main absorption band around 675 nm at low temperature. The spectra in this wavelength region of absorbance changes caused by excitation, charge separation and triplet formation were determined. Here an attempt will be made to identify the Q_y absorption bands of the different chlorophylls and pheophytins present in the reaction-center on the basis of a comparison of these spectra. If this is at all possible, it will justify a posteriori the implicit assumption that exciton interactions between the pigments are not too strong. The spectra will be analysed in terms of gaussian components on a wavelength scale; due to the narrow range studied, conversion to an energy scale does not significantly change the spectral shape.

The triplet state

The most simple spectrum is that of triplet formation (Fig. 2, curve 3). EPR data indicate that the triplet state is localized on a monomeric chlorophyll *a* molecule [22,23]. The absorption spectrum of triplet chlorophyll in this wavelength range is low and flat [24]. If only a monomeric chlorophyll molecule was involved, the difference spectrum upon triplet formation would be expected to consist of the bleaching of a single spectral form of chlorophyll. This is not observed. The maximum is at 680.5 nm and the slope at the short-wavelength side is clearly steeper than that at the long-wavelength side. The absorption spectrum of the sample (Fig. 1) in this spectral region can be described approximately by two 5 nm wide gaussian bands at about 679.5 and 683.5 nm in an amplitude ratio of 3:1, largely independent of what bands are used to fit the absorbance in the 670 nm region (see Fig. 6). The difference spectrum upon triplet formation shows that both the 679.5 nm band and the 683.5 nm band must be affected. In fact at 100% triplet yield both bands would be bleached completely, but in addition the appearance of a new absorption band on the short-wavelength side must be postulated in order to explain the apparently smaller ratio and the shifted maximum. The presence of such a band in the absorption spectrum of the sample can also be visualized directly by adding the triplet difference spectrum to the absorption spectrum of Fig. 1 (not shown). The peak wavelength and amplitude of this band were determined by a nonlinear least squares fit to the measured difference spectrum, assuming that the 679.5 and 683.5 nm components of the absorption

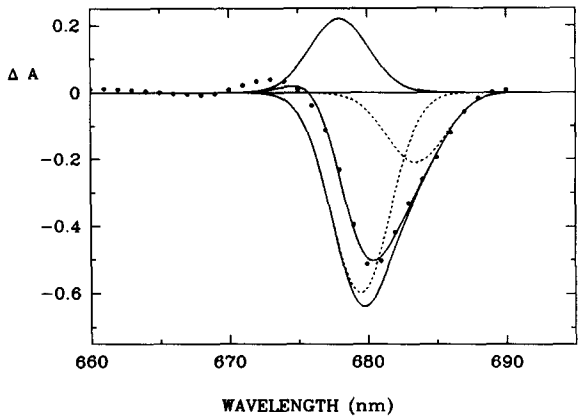

Fig. 4 Gaussian deconvolution of the difference spectrum attributed to P^T minus P (curve 3 in Fig. 2). Peak positions, halfwidths and relative amplitudes: 683.5 nm, 5 nm wide, −0.52; 679.5 nm, 5 nm wide, −1.48; 678.0 nm, 5 nm wide, +0.54.

spectrum are bleached and that the new band should also be a 5 nm wide gaussian. The new band was found to peak at 678 nm, with an amplitude similar to that of the 683.5 nm band (Fig. 4). Subsequent optimization confirmed the 5 nm bandwidth. The small deviations at shorter wavelengths may be due to errors introduced by disregarding higher energy transitions of the pigments. The variation between preparations of this difference spectrum appeared to be due primarily to different amplitudes of the positive 678 nm component.

These observations lead to the conclusion that the 679.5 nm band and the 683.5 nm shoulder together constitute the absorption spectrum of a chlorophyll dimer. Upon triplet formation the exciton interaction is lost and the absorption spectrum is replaced by the sum of the spectra of a triplet monomer (neglected in the fit) and a ground state monomer peaking at 678 nm. This band would then be expected to have half of the combined oscillator strengths of the 679.5 and 683.5 nm bands. This interpretation is analogous to that proposed in Ref. 25 to explain the difference spectrum attributed to P^+ minus P. The 679.5 and 683.5 nm bands indicate an exciton splitting of 85 cm^{-1}, their average red-shift relative to the monomer band may be attributed to an aggregate environmental shift [26] of 75 cm^{-1}, and their 3 : 1 amplitude ratio in first approximation suggests that the Q_y transitions of the two monomers of P make an angle of about 60° [27].

The radical pair

The difference spectrum associated with the formation of the radical pair (Fig. 2, curve 2) is similar to that associated with triplet formation, but contains in addition a bleaching around 675 nm and a small, broad increase on the short-wavelength side. After imposing a 40 nm wide gaussian to approximately describe this broad increase, the additional bleaching was best fitted (Fig. 5) by a 6.5 nm wide gaussian centered at 676.5 nm,

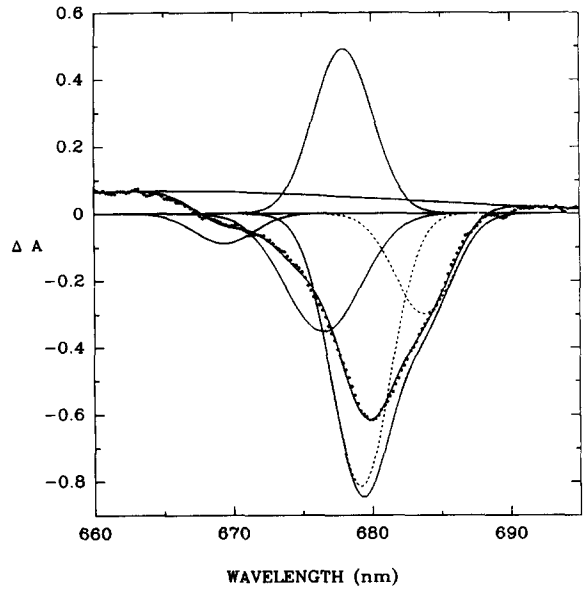

Fig. 5. Gaussian deconvolution of the difference spectrum attributed to P^+I^- minus P I (curve 2 in Fig. 2). Peak positions, halfwidths and relative amplitudes: 683.8 nm, 5 nm wide, −0.54; 679.2 nm, 5 nm wide, −1.46; 678.0 nm, 5 nm wide, +0.88; 676.5 nm, 6.5 nm wide, −0.63; 665 nm, 40 nm wide, +0.12; 669.4 nm, 5 nm wide, −0.16.

with a relative amplitude of about 2/3 that expected for the Q_y band of a chlorophyll a monomer. This outcome is consistent with the assignment of the bleaching to the Q_y band of a pheophytin *a*. The significantly larger width of this band as compared to the chlorophyll bands may be related to the finding by Fragata et al. [28] that the pheophytin Q_y band consists of two nearly degenerate transitions. The broad and flat absorption increase is expected to contain contributions both by the reduced pheophytin and by the oxidized chlorophyll [29] and its approximation by a single wide gaussian may well introduce significant errors. It is not clear if the dip around 669 nm results from such errors, from higher-energy transitions [28] of P and I, or from electrochromic shifts of accessory chlorophylls. These details do not affect the main conclusion that the Q_y band of I is about 6.5 nm wide and peaks near 676.5 nm. Finally, it is noted that a significantly better fit was obtained with a slightly larger exciton splitting of the P bands than in Fig. 4. The variability of the absorption spectra of PS II reaction-center preparations may be due to slight differences in the geometry of the special pair.

Photoaccumulation of I^- is known to cause a main bleaching at longer wavelengths, also in the isolated PS II reaction center [1]. We have confirmed that with the preparation used here (not shown) and conclude that the difference spectrum must be dominated by a blue-shift of P, because no other pigments absorb at longer wavelengths. This interpretation was originally proposed by Ganago et al. on the basis of linear dichroism measurements [30], recently confirmed for isolated reaction-centers [21]. Nuijs et al. [31] proposed that a different chlorophyll was involved, because a band shift was still observed when P was in the oxidized state. However, the shift then observed could be due to the absorption band of the nonoxidised monomer. The identical peak wavelengths we find for this monomer in the presence of I^- and for that left in the ground state in P^T would then be fortuitous, or the difference might be too small to detect in this way.

Accessory pigments

At this stage we can go back to the absorption spectrum and try to locate the Q_y transitions of the remaining pigments. The Q_y bands of one pheophytin and two [6–8], three [1], four [12] or even more [11] accessory chlorophyll molecules have yet to be accounted for. The 'inactive' pheophytin may be spectrally indistinguishable from I [21]. In that case the Q_y bands of all accessory chlorophylls must lie below 675 nm, since essentially all absorbance at longer wavelengths is accounted for by P and both pheophytins. The integrated absorbance in the 660–675 nm range is enough to accommodate four chlorophyll Q_y bands, but must also contain contributions by the pigments having their Q_y bands at longer wavelengths. The second derivative of the absorption spectrum (Fig. 1B) and also the difference spectrum at 10 ps after the flash (Fig. 2, curve 1), which presumably contains contributions by excitation of all pigments, suggest that the Q_y bands of

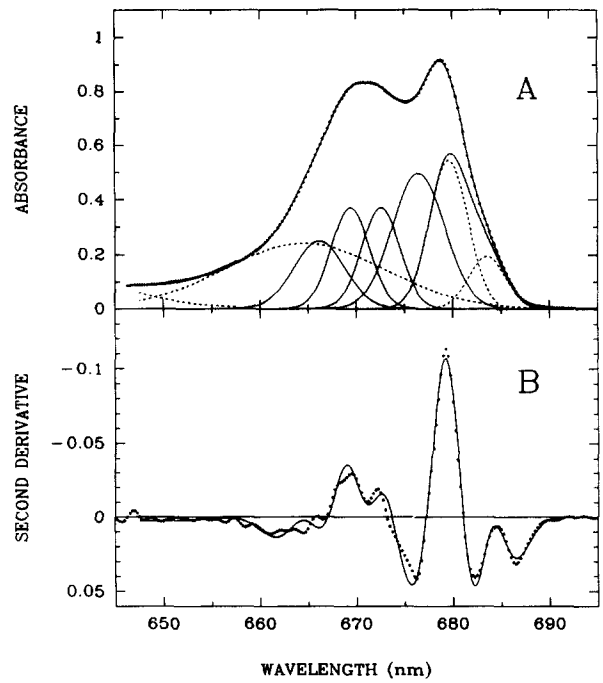

Fig. 6. Gaussian deconvolution of the absorption spectrum (A) and resulting fit of its second derivative (B). The parameters used are given in Table I.

the accessory pigments do not extend below 660 nm. We therefore arbitrarily imposed a 20 nm wide gaussian around 660 nm to account approximately for the higher energy transitions of all pigments together. Furthermore, it was initially assumed that the Q_y bands of the accessory chlorophylls should have a width of 5 nm and a height equal to the average of the 679.5 and 683.5 nm bands. The maxima at 669 and 672 nm in the second derivative spectrum (Fig. 1B) provided an initial guess for their peak positions. With two such bands, however, an acceptable fit could not be reached, but a third band with the same amplitude was too much. An almost perfect fit was obtained when the third band was given the width and amplitude of a pheophytin. Subsequent iterative optimization led to a unique solution with a minimum χ^2 for all parameters and a convincing fit even in the second derivative (Fig. 6 and Table I). Using these more precise values did not significantly improve the fits in Figs. 4 and 5 and may not be justified in view of the different spectral resolution.

The number of accessory chlorophylls cannot be determined from this spectral deconvolution. The 20 nm wide gaussian imposed to account approximately for the combined higher energy transitions of all pigments may contain large errors and the origin of the 666 nm band is not clear. It has been noted by Breton [21] that aging at room temperature specifically increases a 667 nm absorbance with negative LD. The selective bleaching on the long-wavelength side under such conditions suggests that the 667 nm pigment is derived from P. This may not occur after removal of Triton X-100, but it could have happened to some extent during the 16 h separation on the anion-exchange column. We conclude that our preparation possibly contains such a contribution and that only the 669 and 673 nm bands can safely be attributed to 'native' accessory chlorophylls. Alternatively, the 666 nm band may indicate the presence of a third pheophytin (as indicated by the bandwidth and amplitude) or accessory chlorophyll (more likely in view of reported Chl/Pheo ratios) in the native PS II reaction-center. The presence of four accessory chlorophylls, however, is also possible: the absorbance spectrum in this wavelength region could contain an additional Q_y band if the absorbance attributed to the long-wave pigments, P and pheophytin, is much less than the arbitrarily imposed 20 nm wide gaussian would imply.

Deconvolution of the difference spectrum at 10 ps has not been attempted. Presumably it is caused by the disappearance of the ground state absorbance and appearance of stimulated emission from the excited state of all pigments, weighted according to their ground state absorbances at 532 nm and their excited-state lifetimes. The excited state of P probably contributes little to the spectrum, because the formation of P^+I^- takes only 1.4 ps [32]. All excited states are transferred to P, or P^+I^- (both P^+ and I^- are known to be very efficient excitation sinks [33]), within about 40 ps, indicating the absence of disconnected chlorophyll in this preparation.

Finally, we found that the absorption spectrum at 230 K could be fitted by the same parameters as that at 6 K, if all bandwidths were doubled. This fit is clearly not unique, however, and significantly different peak positions of the various Q_y bands at higher temperatures cannot be excluded.

TABLE I

Gaussian components used in the deconvolution of the absorption spectrum in Fig. 6

λ_{max} (nm)	$\Delta\lambda$ (FWHM)	Rel. Ampl.	Assignment
683.6	4.5	0.53	chlorophyll *a* dimer (P)
679.6	4.7	1.47	
676.5	6.4	1.34	two pheophytins *a* (one of which is I)
672.6	4.7	1.00	first accessory chlorophyll *a*
669.4	4.7	1.00	second accessory chlorophyll *a*
666.2	6.3	0.67	inactivation product or 7th pigment
664.6	19.7	0.65	combined higher-energy transitions
626.0	26.4	1.03	chlorophyll vibrational bands

Concluding remarks

We conclude that our spectra can probably be explained by the sum of the spectra of the individual pigments, which would indicate that there is much less interaction between the pigments than in purple bacteria [34]. We found clear evidence for exciton interaction only between the molecules of the special pair. The effect of the presence of 0.05 Triton X- 100 [5] indicates that this interaction is remarkably easily lost or strongly diminished, without preventing photochemical activity. Indications for such a phenomenon have been reported earlier [25]. It should be noted, however, that the difference spectra attributed to oxidation at room temperature [35] or triplet formation at low temperature [20] in isolated PS II core particles are not only more symmetrical but also wider than the low temperature difference spectra reported here and, remarkably, the first reported difference spectrum now known to reflect the oxidation of P at room temperature [36]. A slightly smaller redshift or larger exciton splitting, such that the high energy band of the dimer coincides with the monomer band, would lead to wider, symmetrical difference spectra like those in Refs. 20 and 35. The structure of the special pair in system II is probably unique, suggesting that this may be essential to achieve its other unique property, the extreme oxidizing potential of the pigment.

Acknowledgements

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