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Spectroscopic characterization of a 5 Chl *a* photosystem II reaction center complex

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Abstract

It is now well established that the isolated photosystem II (PS II) reaction center complex in its most stable form binds 6 chlorophyll *a*, 2 β-carotene and 2 pheophytin *a* molecules. By using immobilised metal affinity chromatography, however, it is possible to isolate PS II reaction center particles binding 5 Chl *a* molecules [Vacha et al. (1995) Proc. Natl. Acad. Sci. USA 92, 2929–2933]. In this report we present a number of steady-state spectroscopic characteristics at very low temperature(s) of the 5 Chl preparation (RC-5) and compare those with data obtained for 6 Chl preparations (RC-6). The results confirm the loss of a chlorophyll molecule absorbing at 670 nm in RC-5, and in addition reveal that the shoulder near 684 nm is more pronounced in this preparation than in any other PS II RC preparation. The RC-5 preparation is therefore ideally suited to obtain more information on the nature of the low-energy absorption. Based on the fluorescence and triplet-minus-singlet absorbance-difference data presented in this paper, we propose that all absorption around 680 and 684 nm arises from the weakly excitonically coupled 'core' of the RC-5 complex, and that the remaining peripheral Chl molecule absorbs at 670 nm. Furthermore, from the temperature dependence of the spectroscopic data we conclude that the 684 nm absorption in isolated PS II reaction center complexes contains about equal contributions from the primary electron donor and from the red-absorbing 'trap' states.

Keywords: Photosystem II; Reaction center; Chlorophyll; Pheophytin

1. Introduction

The isolated photosystem II reaction center (PS II RC) complex consists of the so-called D1 and D2 polypeptides and of a number of small protein subunits [1,2]. The amino acid sequences of the D1 and D2 polypeptides show clear homology with those of the L and M subunits of the purple bacterial RC [3],

Abbreviations: Car, carotene; CCD, charge coupled device; CD, circular dichroism; Chl, chlorophyll; HPLC, high-performance liquid chromatography; OD, optical density; Pheo, pheophytin; PS, photosystem; RC, reaction center; RC-*n*, Photosystem II reaction center complex with *n* bound chlorophyll *a* molecules; T − S, triplet-minus-singlet.

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which indicates that both types of complexes are of the same type. The number and way of organization of pigments within both complexes, however, show some important differences. The purple bacterial RC binds 4 BChl, 1 carotenoid and 2 BPheo molecules, while the isolated PS II RC complex in its most stable form binds 6 Chl *a*, 2 β-Car and 2 Pheo *a* molecules [4–6].

Several ideas have been put forward on the organization of the Chl *a* and Pheo *a* molecules in the PS II RC complex (see, e.g., [7,8]). Most of these ideas have in common that in the PS II RC complex 4 Chl *a* and 2 Pheo *a* molecules form the 'core' of the RC in a similar way as in the bacterial RC, but in which the coupling between the 'special pair' molecules is diminished [7,9–11]. In addition there are two extra chlorophylls bound in the periphery of the RC, of which at least one is thought to absorb at 670 nm and to be responsible for 'slow' ($\sim$ 10–30 ps) excitation energy transfer to the core [12–14]. The second extra chlorophyll was either suggested to absorb near 680 nm [8,15], or near 670 nm [7,16].

It has recently been shown that PS II RC particles binding 5 Chl *a* molecules can be isolated using immobilised metal affinity chromatography [17]. In contrast to previously published results on PS II RC complexes which were believed to bind only 4 Chl molecules [18,19], the absorption spectrum of this 5 Chl preparation clearly differs from that of 'standard' 6 Chl preparations in that it has a red-shifted Q_y-absorption band at room temperature (the peak position shifts from 675.5 to 677.5 nm) as well as reduced Chl-absorption near 670 nm. From these first results it was suggested that (one of) the peripheral Chl molecule(s) absorbing around 670 nm is removed in the 5 Chl PS II RC particles. This suggestion was confirmed by transient absorption experiments, which showed a smaller amplitude of the 'slow' energy transfer component from 670 to 680 nm [17].

In this report we present a number of steady-state spectroscopic characteristics at very low temperature(s) of the 5 Chl preparation (RC-5), with the ultimate goal to get a better understanding of the structure and organization of the chromophores in this complex. The results confirm the reduced absorption at 670 nm in the 5 Chl preparation and in addition reveal that the shoulder near 684 nm is more pronounced in this preparation than in any other PS II RC preparation. Evidence is presented that this low-energy shoulder originates, at least in part, from the primary electron donor P680.

2. Materials and methods

Photosystem II reaction centers containing approx. 5 Chl *a* and 1 β-Car per 2 Pheo *a* molecules (RC-5) were isolated from *Pisum sativum* using immobilized metal affinity chromatography as described by Vacha et al. [17]. Reaction centers containing approx. 6 Chl *a* and 1.5 β-Car per 2 Pheo *a* molecules (RC-6), were isolated from spinach CP47-RC complexes [20] by a short Triton treatment as described in Kwa et al. [21]. The pigment content of the reaction centers was determined with two different HPLC-pigment analysis methods [22,23], which were checked to give the same results within the error of the measurement. The purity of all preparations presented in this study was assessed using diode-array-detected gel filtration chromatography [6]. The chromatogram of the RC-5 preparation showed a single peak with a rather straight A_{416}/A_{435} ratio of about 1.30 through this peak (not shown), indicating a single type of complex that has been shown to be a monomer [24] and the complete absence of the core antenna protein CP47 [6]. The A_{416}/A_{435} ratio of 1.30 is significantly higher than the value of 1.20–1.22 observed in Triton-derived RC-6 preparations [6], in agreement with the lower Chl/Pheo ratio in the RC-5 preparation.

Low-temperature steady-state absorption spectroscopy was performed on a Cary 219 spectrophotometer and low-temperature fluorescence spectroscopy was carried out using an imaging CCD camera (Chromex) as detection system and a broad-band 250 W tungsten lamp and 590 nm interference filter as a source of excitation light. Temperature-dependent triplet-minus-singlet (T − S) absorbance difference spectra were measured as described by Kwa et al. [11], using 665 nm excitation light (4.3 mW/cm^2) from a CW dye laser (Coherent 599, DCM dye, spectral bandwidth ~ 2 cm^{-1}) pumped by an Ar^+-laser (Coherent Innova 310). For all low temperature measurements the samples were diluted to an optical density of 0.05–0.1 for the fluorescence experiments or ~ 0.5 for the absorbance-difference experiments in a buffer containing 20 mM Bis Tris,

20 mM NaCl, 10 mM $MgCl_2$ at pH 6.5 (BT-buffer), 0.03% dodecyl maltoside and 70% (w/v) glycerol, and placed in a perspex cuvette ($1.0 \times 1.0 \times 4.0$ cm) which was cooled to the desired cryogenic temperature in a He-bath cryostat (Utreks). Circular dichroism was measured using a special cuvette with quartz windows as described by Kwa et al. [25].

3. Results and discussion

3.1. Absorption spectra at 4K

In Fig. 1A the complete 4K absorption spectrum of a typical RC-5 preparation is shown (full line) together with the spectrum of a RC-6 preparation (dashed line). The spectra were normalized on the amplitude of the Q_x-band of Pheo *a* at 543 nm. The difference spectrum is also shown (dotted line). From the lower absorptions near 450–510 nm and around 670 nm it can be concluded that the spectra differ in their β-Car and Chl *a* contents, which is in agreement with the results of the pigment analysis [17,24]. From the difference between the 4K absorption spectra of the two preparations it can be concluded that a Chl molecule absorbing around 670 nm is removed in the RC-5 preparation, in agreement with the data in Refs. [17] and [24].

Fig. 1B shows an enlargement of the Q_y-absorption region of both spectra, together with their second derivatives. The RC-5 preparation shows considerably less absorption around 670 nm, which can be explained by the removal of a 'blue'-absorbing peripheral chlorophyll molecule on the Cu-affinity column. The second derivative of both preparations peaks at 669.7 nm in this part of the spectrum, which suggests that the RC-5 complex still contains a Chl that absorbs at the same wavelength as the one that is removed. Both preparations show a similar profile peaking at 679.3 nm. The shoulder around 684 nm, however, is more pronounced in the 5-Chl preparation. This feature is also reflected in the second derivative, where it shows a dramatically increased amplitude and a red-shifted peak position (684.4 nm,

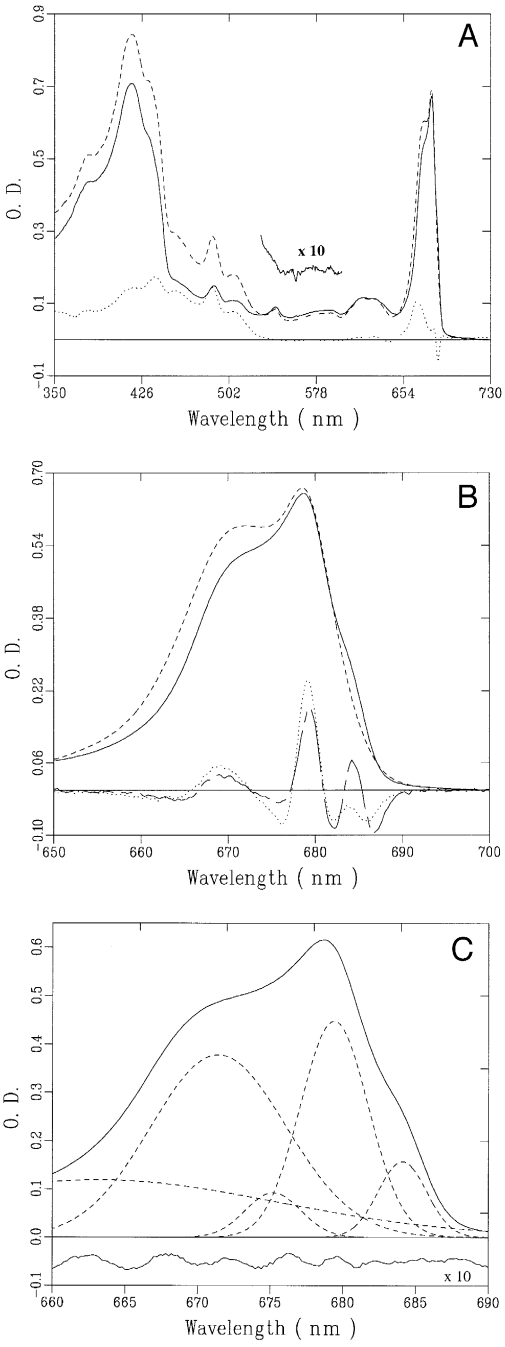

Fig. 1. A: 4K absorption spectra of RC-5 (solid) and RC-6 (dashed), normalized on the Pheo *a* Q_x-band, and their difference spectrum (dotted). The upper spectrum around 550 nm is a 10× enlarged part of the difference spectrum. B: 4K absorption spectra of RC-5 (solid) and RC-6 (dashed), normalized as in A, together with their second derivatives (chain-dashed and dotted, respectively). C: 4K absorption spectrum of RC-5 with the Gaussian components (dashed) resulting from deconvolution (see text and Table 1). The lower spectrum is a 10× enlarged difference spectrum of the fit and the original.

compared to about 683.9 nm in RC-6 – see also Refs. [6,11,26]). These results suggest that the red-shift of the room temperature absorption spectrum of the RC-5 preparation not only originates from the removal of a peripheral Chl peaking near 670 nm, but also from the increased absorption near 684 nm.

Many studies on various types of RC-6 preparations have revealed the presence of a red shoulder in the 4K absorption spectrum [6,8,11,27,28]. The possibility of contamination of RC preparations with the core antenna protein CP47 has seriously hampered the interpretation of the nature of this shoulder (because CP47 also contains 684 nm absorbing states), but now most researchers tend to agree that this shoulder represents a real feature of the PS II RC complex. The shoulder was attributed to the presence of a special, 'linker'-type of antenna Chl [8,15,28] or to a low-energy fraction of the primary electron donor P680 peaking near 684 nm [11]. The rather unusual amplitude of the 684 nm shoulder in the RC-5 preparation makes this preparation ideally suited to obtain more information on the nature of this low-energy absorption.

3.2. Deconvolution of 4K absorption spectra

In Fig. 1C the result of a deconvolution of the 4K absorption spectrum of RC-5 in Gaussian components is shown. We are well aware of the risk of over-interpretation of the results of this kind of deconvolution, as was already stressed in Ref. [6], where a similar approach was used and discussed for several RC-6 preparations. In agreement with the results on the RC-6 preparations, the 4K spectrum of the RC-5 preparation could reasonably well be deconvoluted into 5 Gaussian components. These components include the main bands peaking near 679 and 671 nm, the red shoulder peaking near 683 nm, a band with very little intensity peaking near 675 nm that appeared necessary to obtain a reasonable fit, and a very broad profile of a collection of vibrational bands. In Table 1 the results of the deconvolutions are shown. The amplitude of the 671 nm band is significantly smaller in the RC-5 preparation, which is in agreement with the removal of a 'blue'-absorbing chlorophyll. The position, amplitude and bandwidth of the main band around 680 nm is very similar in the RC-5 and RC-6 preparations. The red-most band, however, peaks 1 nm more to the red in the RC-5 preparation and is more narrow, but has about the same oscillator strength as the corresponding band in the RC-6 preparation.

These results suggest that the increased amplitude of the red shoulder in the 4K absorption spectrum is caused by a red-shift and narrowing of the 684 nm absorption band, but not by a larger amplitude of this absorption.

3.3. Circular dichroism spectra

Fig. 2 presents CD spectra of the RC-5 preparation at room temperature (solid line) and 77K (dashed line). The spectra were normalized on the amplitude of the positive peak near 680 nm. The spectra show positive chirality peaking at 681 nm (293K) and 680 nm (77K), respectively, and a negative contribution around 666 nm. The spectra are comparable to spectra of RC-6 preparations reported in earlier publica-

Table 1
Results of deconvolution into Gaussians of the 4K absorption spectra

RC-6	683.0 [a]	679.1	674.6	671.3	663.2	wavelength (nm)
	60	56	39	130	244	width (Å)
	0.1133	0.3950	0.0413	0.4682	0.1371	height
	6.8	22.1	1.6	60.9	33.5	width × height
RC-5	684.1	679.4	675.2 [a]	671.4	663.2 [a]	wavelength(nm)
	39	56	45	109	269	width (Å)
	0.1572	0.4513	0.0919	0.3792	0.1163	height
	6.1	25.3	4.1	41.3	31.3	width × height

[a] The wavelength of the red-most absorption band was frozen at the same wavelength in the two preparations in order to stress the differences between the preparations. Inclusion of this wavelength as a free fit parameter usually resulted in slightly different fits which, however, did not differ significantly in fit quality.

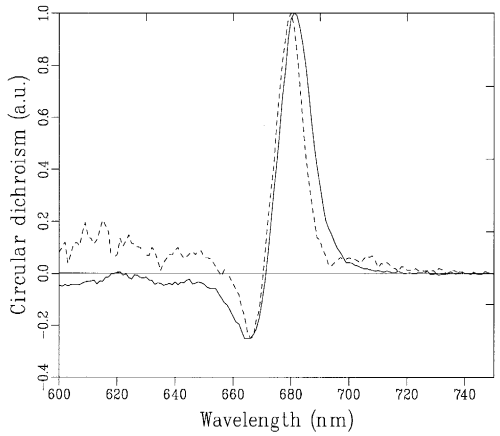

Fig. 2. Circular dichroism spectrum of RC-5 measured at room temperature (solid) and 77K (dashed).

tions [9,29–31], except for the amplitude of the negative peak at 666 nm, which is approx. 25% smaller in the RC-5 preparation. These results suggest that the structure of the (more or less) excitonically interacting RC core pigments is very similar in the RC-5 and RC-6 preparations, in agreement with the idea that a peripheral Chl peaking at 670 nm is absent in the RC-5 preparation.

3.4. Fluorescence spectra

The 4K emission spectrum of the RC-5 complex is characterized by a narrow band peaking at 684.0 nm and a fwhm of 7.9 nm (not shown). It differs slightly from the spectrum of RC-6, which peaks at 683.6 nm [32,33] (see also below) and has a fwhm of −8.5 nm. For all temperatures below 50K the width of the spectrum of the RC-5 preparation appeared to be about 10% smaller (Fig. 3A). Also of note is that at all temperatures between 4K and 275K the emission maximum peaks approx. 0.5 nm further to the red (Fig. 3B) compared to the normal RC-6.

The 4K emission originates from the so-called ‘trap’ pigments or states [32], which are degenerate with P680, and are unable to transfer excitation energy to P680 at very low temperatures. Under these conditions, the excitation energy has a much higher probability to be emitted as fluorescence. The lifetime of this fluorescence for RC-6 was recently esti-

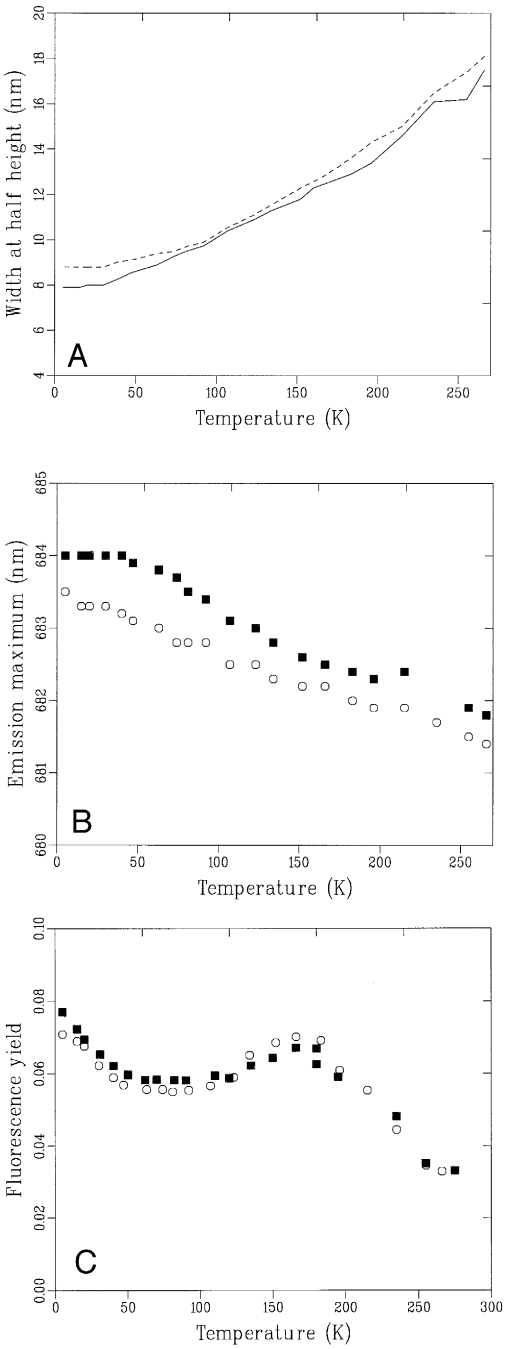

Fig. 3. A: FWHM (nm) as a function of temperature (4–275K) of the emission spectra of RC-5 (solid) and RC-6 (dashed). B: Emission maximum as a function of temperature (4–275K) of RC-5 (closed squares) and RC-6 (open circles). C: Fluorescence quantum yield as a function of temperature of RC-5 (closed squares) and RC-6 (open circles), obtained by integrating the emission spectra from 650 to 700 nm as described in detail in [32].

mated by hole-burning techniques to be 4 ns, while the distribution of the pigments responsible for this 4 ns lifetime was shown to peak at 681.8 nm [34].

Our results clearly indicate that the narrowing and red-shift of the 684 nm shoulder in the 4K absorption spectrum of the RC-5 preparation is accompanied by a narrowing and red-shift of the emission spectrum at various temperatures. This suggests that the 684 nm shoulder is largely responsible for the 4K emission, in agreement with suggestions by [28] and [15]. In the following we will show, however, that also the primary electron donor P significantly contributes to this low-energy shoulder, in agreement with earlier suggestions by [35] and [11].

3.5. Fluorescence quantum yields

By measuring the fluorescence quantum yield as a function of temperature the interactions between the different pigment pools can be studied. At 4K we expect the excitation to be trapped on the chlorophyll with the red-most absorption. If this chlorophyll is not part of the primary electron donor P680, the excited state cannot escape via charge separation and will decay to a large extent via fluorescence (see above). The yield of the fluorescence as a function of temperature will therefore provide an indication whether or not such trapping chlorophylls are present, and in addition provides information on the energetics of the uphill energy transfer to P680.

Fig. 3C shows that the temperature dependence of the fluorescence quantum yield of RC-5 (closed squares) is very similar to that observed for RC-6 (open circles). Upon lowering the temperature from about 160K to 80K, the emission yield decreases. This can qualitatively be explained by the shift of the equilibrium between the radical pair and the excited state towards the radical pair. When the temperature is lowered even further (from 60K to 4K), an increase in emission yield occurs, caused by the increased contribution of the 'trap' pigments [32]. Raising the temperature above 160K causes the yield to decrease again which can be explained by a temperature dependence of the excited state decay via internal conversion [36].

The similarity of the fluorescence quantum yields as a function of temperature in the RC-5 and RC-6 preparations suggests that the relative amounts and energetics of P680 and the 'trap' pigments are the same in both types of preparations. This suggests that not only the 'trap' states are narrowed and red-shifted in the RC-5 preparations, but also P680. More information on the spectral distribution of P680 was obtained by triplet-minus-singlet (T − S) absorbance-difference spectroscopy (see below).

We note that we found, as yet, only one type of PS II RC preparation with a different temperature dependence of the steady-state emission than observed in Fig. 3C. Long-time Triton X-100-treated RC-6 preparations revealed a much higher fluorescence yield at very low temperatures than the other preparations [6], suggesting that in this type of preparation the distribution of P680 is blue-shifted compared to that of the 'trap'-pigments. These preparations showed a decreased 684 nm shoulder in the 4K absorption spectrum.

3.6. Triplet-minus-singlet absorbance-difference spectra

Fig. 4A shows triplet-minus-singlet (T − S) absorbance difference spectra at 4K of RC-5 and RC-6 obtained upon non-selective excitation at 665 nm. The spectra were smoothed using a polynomial filter and normalized on their negative maximum ($\sim$ 680 nm). They are characterized by a relatively broad bleaching peaking near 680 nm and, in case of RC-5, a very pronounced shoulder at 684 nm. Due to the strong contribution of the feature at 684 nm, the T − S spectrum of RC-5 shows a much broader bleaching than observed before for any other preparation [6,11,15,27].

By means of fluorescence and T − S quantum yield measurements on RC-6 preparations, it was suggested that at 4K about 75% of the observed absorbance-difference signal arises from the spin-polarized triplet of P680 and about 25% from the triplet of the 'trap' [32]. These numbers should also hold for the RC-5 preparations, because the temperature dependencies of the emission yields are the same (see above). We therefore conclude that a significant part of the bleaching at 684 nm in the T − S spectrum originates from a low-energy fraction of the primary electron donor, which we designate P684.

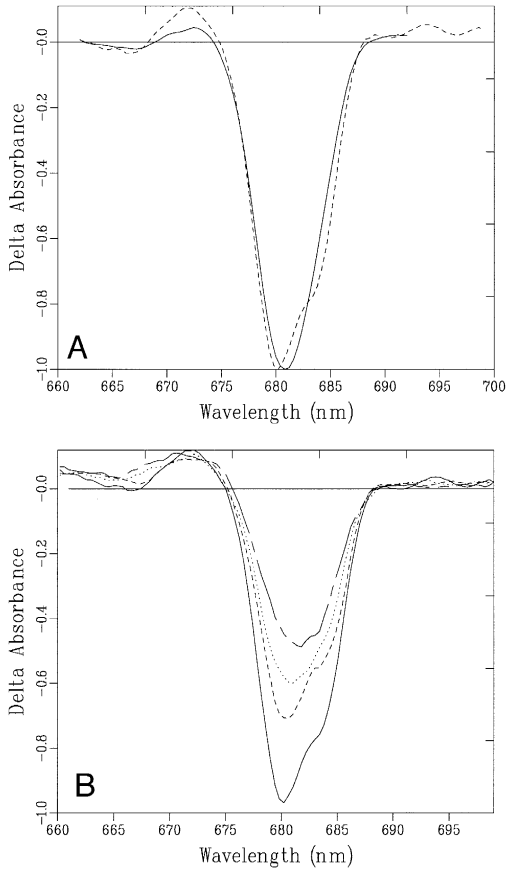

Fig. 4. A: Triplet-minus-singlet absorption difference spectra of RC-5 (solid) and RC-6 (dashed) measured at 4K. B: Triplet-minus-singlet absorption difference spectra of RC-5 measured at T = 4K (solid line), l5K (dashed line), 30K (dotted line) and 56K (chain-dashed line).

3.7. Temperature dependence of triplet-minus-singlet spectra

We also recorded T − S spectra of the RC-5 preparations at 15, 30 and 56 K. In Fig. 4B we show that upon raising the temperature the shape of the RC-5 T − S spectrum changes from two separated negative maxima peaking at 680.2 and 683.8 nm at 4K to one broad bleaching peaking at 681.8 nm at 56K.

These types of experiments were also performed by Chang et al. [15] on long-time Triton X-100 treated RC-6 preparations, and by us on more gently treated RC-6 preparations [6]. Chang et al. observed a clear narrowing of the T − S spectrum upon raising the temperature from fwhm ∼ 5.6 nm at 4K to ∼ 5.3 nm at 30K. The narrowing was conveniently explained by a decreased contribution of red trapping pigments at higher temperatures as a result of an increased possibility of uphill energy transfer to P680 [15]. The narrowing was, however, not observed in the T − S spectra of the more gently treated RC-6 preparations (which showed a fwhm of 6.7 nm at 4K and 6.9 nm at 30K [6]), and is also not observed in the T − S spectra of the RC-5 preparations (Fig. 4B), which show a fwhm of 7.6 nm at 4K and 30K. For an explanation we first note that also in the latter preparations the relative contribution of P in the T − S spectrum increases upon raising the temperature. This notion is based on the results from the temperature dependence of the fluorescence quantum yields. With this in mind, the absence of a narrowing of the T − S spectrum upon raising the temperature suggests that the distribution of P680 is not blue-shifted compared to the distribution of the trap in these latter preparations.

It has recently been suggested that the ‘core’ chlorophylls and pheophytins of the PS II RC are weakly excitonically coupled, and that, depending on the specific inhomogeneous disorder in every individual reaction center complex, P680 actually consists of a varying number of these core pigments [7]. For many possibilities of disorder, two nearly degenerate exciton states were found, which were roughly delocalized over the two ‘arms’ of the reaction center. We suggest, in line with Refs. [16] and [37], that one of these lowest exciton states includes the P680 multimer, and that the other lowest exciton state is responsible for the ‘trap’. This idea easily explains the similar spectroscopic features of the P680 and trap states (observed in T − S and fluorescence experiments, respectively) in terms of peak position and bandwidth in the various RC-5 and RC-6 preparations.

In summary, we apply a model for RC-6 in which the reaction center consists of a ‘core’ of 4 Chl *a* and 2 Pheo *a* molecules similar to that in the purple bacterial RC [7] and of two additional peripheral Chl molecules. Our results indicate that RC-5 lacks one of the peripheral Chl molecules and that the two peripheral Chl molecules of RC-6 both absorb at 670 nm. The finding that the second derivative of the RC-5 preparation still shows a clear band at 669.7 nm (Fig. 1B) is in line with this idea. Also the result of Ref. [17] that half of the slow (∼ 30 ps) energy

transfer from 670 nm to 680 nm is still observed in RC-5 can conveniently be explained in terms of this model. Furthermore, we suggest that, in contrast to the proposals in Refs. [28] and [8], all absorption around 680 and 684 nm arises from the 'core' of the reaction center complex and that the 684 nm absorption in isolated PS II reaction center complexes contains about equal contributions from the primary electron donor and from the red-absorbing 'trap' states.

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