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A comparison of the photochemical activity of two forms of Photosystem II reaction centre isolated from sugar beet

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Abstract

Both time-resolved fluorescence and absorption measurements have been conducted on two different forms of Photosystem II reaction centre isolated from sugar beet. One form, called RC IIa, contained 6 chlorophylls and 2 β-carotenes per 2 pheophytins, while the other, called RC IIb, contained 4 chlorophylls and 1 β-carotene per 2 pheophytins. Single photon-counting fluorescence decay obtained from the two preparations showed similar charge recombination fluorescence lifetimes which could be resolved into two components of 46.1 and 14.2 ns. Analysis of the amplitude of the fluorescence of the fast component of 5.6 ns, which largely originates from non-functional chlorophyll, gave an estimate of the relative activity for RC IIb which was only a 5.5% lower compared to that of RC IIa. This small relative difference in photochemical activity was also confirmed by measuring the extent of primary charge separation activity using flash induced absorption spectroscopy. In this case the amplitude of the long-lived component, attributed to primary radical-pair formation and recombination, was 16% lower in RC IIb as compared with RC IIa. When the secondary electron transfer activity of the two forms of reaction centre were measured using $MnCl_2$ and silicomolibdate as electron donor and acceptor respectively, RC IIb was 16% less active than RC IIa. From the data we conclude that the removal of 2 chlorophylls and 1 β-carotene molecules from the isolated Photosystem II reaction centre only slightly impair its functional activity with respect to primary charge separation. This conclusion seems to suggest that photochemically active isolated reaction centres of Photosystem II and purple bacteria can have the same minimum pigment stoichiometry of 4 chlorophylls and 1 carotenoid per 2 pheophytins.

Key words: Electron transfer; Fluorescence; Photosystem II; Reaction center; Sugar beet; Time-resolved

1. Introduction

The reaction centre of Photosystem II (PS II), consisting of the D1 and D2 polypeptides, the cytochrome (Cyt) *b*-559, and the *psbI* gene product has been isolated from several sources [1–4]. The stoichiometry of chromophores associated with this protein complex has been a subject of disagreement in the literature. In the first preparations [1,2] a content of 4–5 chlorophylls (Chl), 1 carotenoid and 2 pheophytins (Pheo) per complex was determined as constitutive of the PS II reaction centre of plants. These early preparations of the isolated reaction centre were, however, labile and unstable showing a high susceptibility to photodynamic damage [5,6]. Later on, with the introduction of milder detergents into the purification protocols (i.e., β-dodecyl maltoside and octyl glucopyranoside) a more stable form of the D1-D2-Cyt *b*-559 complex was obtained [7,8]. In this case the pigment stoichiometry was found to be 6 Chl and 2 β-carotenes per 2 Pheo [9,10].

Recently, a procedure for isolating the D1-D2-Cyt *b*-559 complex from sugar beet was reported [11,12], showing that two forms could be obtained, one binding 6 Chl and the other 4 Chl per 2 Pheo (called hereafter RC IIa and RC IIb respectively). It was also shown in that paper that the removal of the 2 Chl was accompa-

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Abbreviations: Chl, chlorophyll; Cyt, cytochrome; Pheo, pheophytin; PS, Photosystem; P680, the special Chl of the Photosystem II reaction centre; RC, reaction centre; SiMo, silicomolibdate.

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nied by a loss of one of the two β-carotene molecules usually found in the more Chl rich complex. The resulting pigment stoichiometry of 4:1:2 for Chl, β-carotene and Pheo is similar to that found in the isolated reaction centre of purple bacteria [13–15]. Thus, the question arises as to the significance, if any, of the two extra Chl and a second carotenoid in the preparation of PS II reaction centre generally used in several laboratories. In this paper we have set out to investigate this question by using time-resolved absorption and emission spectroscopy to probe primary charge separation and recombination in the two forms of the PS II reaction centre isolated from sugar beet. We have also tested the ability of the two types of complexes to perform secondary electron transfer.

2. Materials and methods

Preparation of the D1-D2-Cyt b-559 complex

The RC IIa and RC IIb complexes were isolated from sugar beet (*Beta vulgaris L.* cv. Monohill) essentially as in Nanba and Satoh [1] for spinach, with some modifications as reported by Montoya et al. [11,12]. After isolation, the material was immediately subjected to detergent exchange from 0.05% Triton X-100 to 2 mM β-dodecyl maltoside [7,8]. To this end, the reaction centre preparations from the first ion-exchange column were diluted four times with 50 mM Tris-HCl (pH 7.2), and loaded onto a small (1.6 × 3 cm) Fractogel TSK-DEAE 650S column (Merck). The column was washed with a buffer containing 50 mM Tris-HCl (pH 7.2), and 2 mM β-dodecyl maltoside before the reaction centre complex was eluted with 145 mM NaCl in the same buffer. No loss of pigments was detected during the exchange of detergent. When necessary, the reaction centre complexes were concentrated with an Amicon ultrafiltration cell using a PM-10 exclusion membrane. These procedures were carried out at 4°C in the dark and did not bring any changes in the optical absorption spectra of the preparations which were measured using a Shimadzu UV/PC-2101 spectrophotometer in 1-cm pathlength cuvettes. The concentrated samples were stored at 77 K until used.

Time-resolved spectroscopy

Time-resolved fluorescence decays were measured using a single photon-counting apparatus as previously described [16]. This system consisted of a mode-locked Coherent Antares YAG laser synchronously pumping a cavity-dumped rhodamine 6G dye laser. This provided a 3.7 MHz train of 8 ps pulses. Samples were illuminated at 630 nm with an average power of 20–30 mW and approximately 2.3 in 10^5 of the reaction centres present in the illuminated volume were excited by each laser pulse. Fluorescence decays were collected up to 40000 counts in the peak channel. Decays were measured over a 6-nm bandwidth at 682 nm using a Chl concentration of 10 μg ml^{-1} for RC IIa and 2 μg ml^{-1} for RC IIb.

The fluorescence decays were analyzed as previously reported [17] using a Marquardt fitting algorithm assuming a multiexponential decay kinetics. The quality of the fits was judged using a reduced χ^2 criterion and plots of the weighted residuals. Data sets were analyzed individually and globally to fit three or four exponentials. Absolute fluorescence quantum yields were estimated for each sample as previously described [16,17].

Transient absorption changes were measured with the flash spectrometer described previously [16,17]. The 337 nm excitation flashes were produced by a N_2-laser at a repetition rate of 3 Hz. The pulse duration was 800 ps and contained 0.45 mJ per pulse. The optical pathlength was 1 cm and data was the average of 64 flashes. The probe beam was provided by a 35 mW laser diode (Mitsubishi ML5415 N), with the wavelength centred at 820 nm and detected with a silicon photodiode (EG&G FN 100) with a gated bias connected to a homebuilt amplifier incorporating a 4.7 μs high pass filter. Transient absorption decays were analyzed as previously reported [16,17]. One lifetime was fixed at 4.7 μs to represent the decay of the P680 triplet while all other parameters are free-running. The lifetime of the P680 triplet in the absence of oxygen is about 1 ms [17] but is observed as a 4.7 μs component because of the use of a 4.7 μs high pass filter. Deconvolution of the transient absorption data was accomplished using the instrument response function and gave a time resolution of 1 ns with an accuracy of ±1 ns. Both time-resolved emission and absorption measurements were conducted on samples at 4°C which were made anaerobic by the inclusion of a glucose/glucose oxidase trap [7,8].

Steady-state fluorescence

Emission spectroscopy of both preparations was carried out at 4°C in the dark using a Perkin-Elmer LS-50 fluorimeter with an excitation and emission bandwidth of 2 nm. The spectra were recorded in buffer 60 mM Tris-HCl (pH 8.0), 2 mM β-dodecyl maltoside and a glucose/glucose oxidase trap [7,8]. Samples were excited at 630 nm. Absolute steady-state fluorescence yields from both kinds of reaction centres were obtained integrating over the fluorescence band from 640 nm to 780 nm and by comparing with a Chl standard of known quantum yield [16].

Secondary electron transfer

Relative electron transfer activities in steady-state were assayed at 4°C using 1 mM $MnCl_2$ and 200 μg ml^{-1} SiMo as artificial electron donor and acceptor,

respectively. The reduction of SiMo was monitored by observing absorbance increases at 600 nm, with a Shimazdu UV-3000 double beam/dual wavelength spectrophotometer, shielding the phototube with a Schott BG18 cutoff filter. The RC IIa and RC IIb suspensions diluted to 0.1125 μg Chl ml^{-1} in 50 mM Tris-HCl (pH 8.5), were illuminated from the top with a slide projector. The actinic light was filtered through a Schott RG665 cutoff filter and a Schott KG3 heat filter. The intensity of the actinic light at the sample was about 1200 μE m^{-2} s^{-1}, which was almost saturating for the low level of Chl used. The concentration of reduced SiMo over a period of illumination was calculated using an extinction coefficient of 4.8 mM^{-1} cm^{-1} [8]. Chl levels were determined by the method of Arnon [18]. All measurements were made at 4°C.

Table 1
Time-resolved fluorescence lifetimes, amplitudes and origin of each component in both types of D1-D2-Cyt *b*-559 complex

Component	RC IIa τ (ns)	Relative yield (%)	RC IIb τ (ns)	Relative yield (%)	Origin of each component
F1	46.1	15.9	46.1	4.6	Charge recombination
F2	14.2	15.0	14.2	6.2	Charge recombination
F3	5.6	65.5	5.6	84.0	Non-functional Chl
F4	1.3	3.6	1.3	5.2	No physical meaning in the collection time scale

Relative fluorescence yields were calculated as the product of the amplitudes and lifetimes of various components.

3. Results

The two forms of isolated PS II reaction centre, RC IIa and RC IIb, were checked by HPLC and pheophytinization to contain the pigment levels previously reported [11,12] before being subjected to the functional analysis detailed below.

Time-resolved fluorescence of RC IIa and RC IIb was recorded in a nanosecond time-scale. The fluorescence decays from each type of reaction centres are depicted in Fig. 1. The analysis of the decays were done individually for each data set looking for the best fit by three or four exponentials [17]. Values of χ^2 below 1.5 with random residuals (as obtained in Fig. 1A and B in S.Dev. boxes) were considered to indicate good fittings. Both type of reaction centres showed a good fit to four exponentials, giving similar lifetimes. An example of the lifetimes obtained in these fits for each reaction centre were 47.9, 11.6, 5.0, and 0.5 ns for RC IIa and 41.5, 11.5, 5.0, and 1.0 ns for RC IIb. In order to be able to compare properly the amplitudes of the different components of both types of reaction centres all the data sets were reanalyzed globally, obtaining the best fit to four exponentials with lifetimes 46.1, 14.2, 5.6, and 1.3 ns. Global analysis [19] reduced the effects of exponential correlation and improved the accuracy of parameters extracted from data. Finally, with these lifetimes assigned to the two types of reaction centre the decays were analyzed again to calculate the amplitude of each component (Table 1). As discussed previously [17], the two longer lived components 46.1 and 14.2 ns are adscribed to radical-pair recombination process and the 5.6 ns to non-functional Chl. In the work by Booth et al. [17], the charge-recombination kinetics of the primary radical pair were demonstrated to be multiexponential and to exhibit at least two components of approximately 50 and 20 ns. The 1.3 ns component has no demonstrable physical mean-

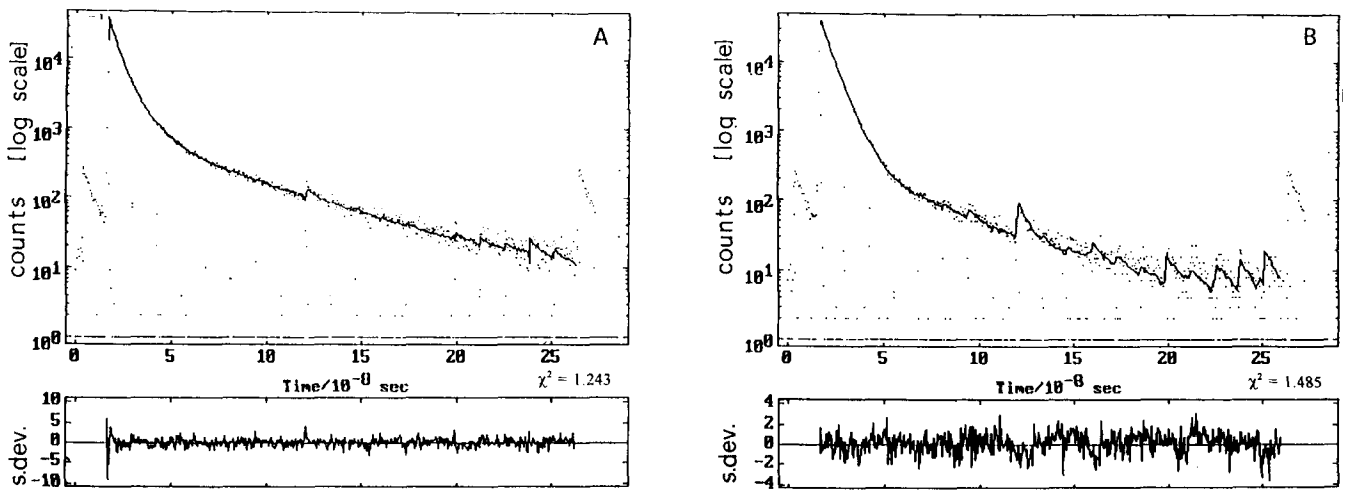

Fig. 1. Typical fluorescence decays from RC IIa (A) and RC IIb (B) forms at 277 K measured with a 6-nm bandwidth at 683 nm. The χ^2 value and residuals for the exponential fit to the data are shown. In the decays the dots correspond to the data and the solid line to the fit.

Table 2
Relative activity of the two different reaction centres, determined by measuring the % of non-functional Chl (Chl uncoupled from the energy transfer) estimated from the data of Table 1 and the absolute quantum yields of each preparation (see [16])

	RC IIa (6 Chl/2 Pheo)	RC IIb (4 Chl/2 Pheo)
Non-functional Chl	8	12.5
Activity (%)	92	87.5
Relative activity (%)	100	94.5

Table 3
Time-resolved flash absorption lifetimes (τ) and origin of each component

Component	RC IIa (6 Chl/2 Pheo)	RC IIb (4 Chl/2 Pheo)	Origin of each component
A1	2.4±0.4 ns	3.2±0.3 ns	see [16]
A2	15.0±1.8 ns	19.2±6.7 ns	Radical pair recombination
A3	57.4±1.6 ns	58.0±5.2 ns	Radical pair recombination
A4	4.7 μs	4.7 μs	P680 triplet

ing on this data collection time-scale [17]. It is possible to estimate the level of non-functional Chl (i.e., Chl uncoupled from the energy transfer) from the fluorescence yield of the longer lived components and the 5.6 ns component. The calculations require a measurement of the absolute fluorescence quantum yield of each reaction centre preparation which were determined by comparing to 0.32 fluorescence quantum yield of Chl *a* in diethyl ether [16]. As can be observed in Table 2, these calculations indicate that RC IIa and RC IIb preparations contain, as an upper limit, 8 and 12.5% non-functional Chl, respectively. The calculations indicate that RC IIb is about 94.5% as active as RC IIa (see Table 2). Others have also conducted time-resolved fluorescence measurements using isolated PS II reaction centre and detected long-lived recombination fluorescence [20–22]. These studies, however, either used samples with low yields of the long-lived component [20] or focussed attention on the very fast components of the decay [21,22] and there has been some disagreement in the interpretation of the data [21].

Transient absorption measurements were made with

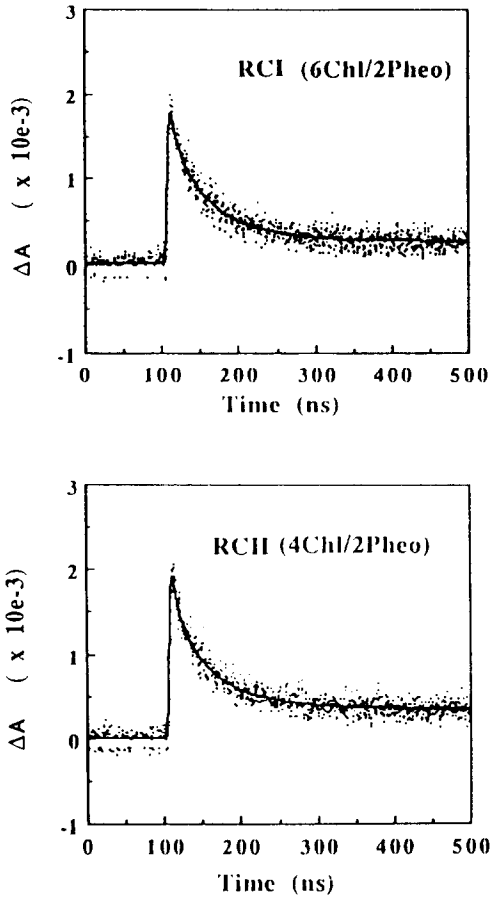

Fig. 2. Typical decays of transient absorption changes at 277 K recorded at 820 nm using either RC IIa and RC IIb preparations. The reaction centre complexes were in buffer containing 50 mM Tris-HCl (pH 7.2), and 2 mM β-dodecyl maltoside.

the flash photolysis apparatus described in [16,17,23]. Fig. 2 shows the time-resolved absorption curve of RC IIa and RC IIb preparations subjected to 800 ps actinic flashes generated by the N_2-laser. The observed decay was fit to four exponential components having lifetimes of 2.4 ns, 15.0 ns, 57.4 ns, and 4.7 μs for RC IIa and 3.2 ns, 19.2 ns, 58.0 ns, and 4.7 μs for RC IIb (Table 3). We ascribe the 15–20 ns and 55–60 ns components to the radical-pair recombination [17]. This shows a good agreement between transient absorption and fluorescence $P680^+Pheo^-$ decay kinetics. The 4.7 μs compo-

Table 4
Relative radical-pair yield of RC IIa and RC IIb forms from sugar beet, calculated from the amplitude of the component A2 and A3, assigned to primary radical-pair recombination

	[Chl] (μg/ml)	[RC] (μM)	Amplitude of A2 and A3 (ΔA)	Radical pair [a] $[P^+I^-]$ (μM)	Radical pair per RC (ratio)	Relative radical pair yield (%)
RC IIa	2.67	0.498	0.0022	0.177	0.355	100
RC IIb	2.24	0.625	0.0023	0.185	0.297	83.6

[a] Considering that the extinction coefficient of P^+I^- at 820 nm is 12.4 $mM^{-1}cm^{-1}$ [24,25].

nent corresponds to the decay of the P680 triplet which has a true lifetime of about 1 ms under these conditions [23] but is limited by the band pass of the electronic filter used (see Section 2). The faster component (approximately 2 ns) also has been observed in reaction centres from pea [17], and its origin is unknown. Table 4 documents the amplitude of the components assigned to the radical pair (component A2 and A3) and indicates again that RC IIb is 83.6% as active as RC IIa in performing primary charge separation. However, during the course of these transient absorption experiments, which involved repetitive and strong flashing with the N_2-laser, we observed that the RC IIb preparations were slightly less stable than the RC IIa samples.

Analysis and comparison of the fluorescence quantum yields in both kinds of reaction centres isolated from sugar beet with reaction centres from pea is shown in Table 5. Some of the reaction centres from pea were partially damaged by treatment with white light of intensity 500 $\mu E\ m^{-2}\ s^{-1}$ during 15 min at 4°C. As is shown in Table 5, the absolute fluorescence quantum yield of the whole pea RC preparation is increased when it is subjected to damage. However, sugar beet RC IIb did not show such a significant increase with respect to RC IIa. When the absolute fluorescence quantum yield of total charge recombination ϕ_{CR} was calculated for each sample, we observed that the ratio of ϕ_{CR} for pea (pea RC/pea RC damaged) was 1.2, whereas the ϕ_{CR} for sugar beet (RC IIa/RC IIb) was 2.4. In contrast, the ratio of the activity (pea RC/pea RC damaged, RC IIa/RC IIb) for each type of reaction centre, deduced from the amount of Chl functionally coupled (see Table 5), has approximately the same value, 1.1.

It is possible to calculate the equilibrium constants for the primary charge separation process $P680^* \rightleftharpoons P680^+Pheo^-$ for both RC IIa and RC IIb at 277 K from the observable parameters as follows [16,17]:

$$K = [P680^+Pheo^-]/[P680^*] = \tau_1/(\phi_{CR} \cdot \tau_0)$$

where τ_1 is the lifetime of charge recombination fluorescence, ϕ_{CR} is the quantum yield of charge recombination fluorescence and τ_0 is the natural lifetime of P680* which is taken as 19 ns [16]. In this way, we obtained an equilibrium constant for the components responsible for the primary charge separation (46.1 and 14.2 ns components) of 202 and 62 for RC IIa, and 485 and 149 for RC IIb. Free energy of primary charge separation of RC IIa and RC IIb can be calculated from their respective equilibrium constants using the following equation:

$$\Delta G = -k_b \cdot T \cdot \ln(K)$$

where k_b is the Boltzmann constant and T is the temperature, which resulted in $\Delta G = -0.125$ eV and -0.097 eV for RC IIa and -0.145 and -0.117 eV for RC IIb.

To compare further the two preparations of D1-D2-Cyt *b*-559 complex isolated from sugar beet, the activity of secondary electron transfer was measured in steady-state conditions. Table 6 displays the activity using $MnCl_2$ and SiMo as artificial electron donor and acceptor, respectively. RC IIb was more active on a Chl basis, but this value has to be corrected for its lower Chl content compared to RC IIa. Considering

Table 6
Secondary electron-transport activity of both types of preparation RC IIa and RC IIb from sugar beet

	μeq.e$^-$ (μmol Chl)$^{-1}$ h^{-1}	μeq.e$^-$ (μmmol RC)$^{-1}$ h^{-1}	Rel. activity (%)
RC IIa	2523	15138	100
RC IIb	3181	12724	84

Table 5
Comparative fluorescence quantum yields in the different PS II RC

Sample	λ_{max} red band (nm)	Absolute fluorescence quantum yield of the whole sample ϕ_F	Absolute fluorescence quantum yield of total charge recombination ϕ_{CR} [a]	Quantum yield of free chlorophyll ϕ_{FChl} [b]	Activity (%)
Pea RC	676	0.038	0.016	0.019	94
Pea RC damaged	674	0.073	0.013	0.054	83
RC IIa	675.6	0.040	0.012	0.026	92
RC IIb	674.8	0.048	0.005	0.040	87.5

The reaction centres isolated from sugar beet with two different pigment compositions are compared with the normal and partially photodamaged reaction centres isolated from pea. The maxima of the fluorescence spectra were 683, 679, 682, and 681.5 nm for pea RC, pea RC partially damaged, RC IIa, and RC IIb preparations, respectively.

[a] ϕ_{CR} = (Relative yield F1 + Relative yield F2) · ϕ_F.

[b] ϕ_{FChl} = (Relative yield F3) · ϕ_F (If ϕ_F of Chl is 0.32, a ϕ_F of free Chl of 0.040 corresponds to 12.5% of free Chl and therefore to 87.5% of coupled Chl).

the activity on a reaction centre complex basis, the RC IIb showed an activity of about 84% compared to RC IIa.

4. Discussion

The photochemical activity of two different forms, RC IIa and RC IIb, of the D1-D2-Cyt *b*-559 complex isolated from sugar beet [11,12] has been determined using three independent measurements. Both preparations maintained comparable activities, despite their different pigment contents. They showed similar time-resolved fluorescence and optical absorption components. We, therefore, conclude that the two additional Chl and the additional β-carotene present in the RC IIa compared to the RC IIb form are not required for primary charge separation to occur. The fact that RC IIb contains 4 Chl and 1 carotenoid per 2 Pheo gives a direct correspondence to the pigment stoichiometry of the reaction centre of purple bacteria [13–15].

Together with these similarities, a difference can be seen between RC IIa and RC IIb when the absolute quantum yield of charge recombination fluorescence is determined (Table 5). The level of charge recombination fluorescence (ϕ_{CR}) in RC IIb is too low (0.005) to be due simply to loss of activity (Table 5). This observation indicates a shift in the equilibrium P680* $\rightleftharpoons$ $P680^+Phe^-$ towards the radical pair in RC IIb. This is the result one would expect, as the equilibrium constant depends not only on ΔG but also on the number of electronic states involved. Thus, a change in the number of chromophores which are in energy transfer equilibrium with P680* will change the equilibrium constant. Indeed, RC IIa contains 6 Chl and 2 Phe, and RC IIb contains 4 Chl and 2 Phe [11]. P680 takes up 2 Chl, so RC IIa has 4 'antenna Chl' while RC IIb has 2 'antenna Chl'. Thus, the equilibrium constant would differ by a factor of 2. Our observed change in the equilibrium constant was 2.4 (Table 5 shows the charge recombination fluorescence quantum yields as 0.012 for RC IIa and 0.005 for RC IIb, a ratio of $0.012/0.005 = 2.4$). Of course, one must realize that the correct way to estimate such an equilibrium constant should really take account of factors such as oscillator strength, pigment coupling and other factors such as the spectrum of both of the Pheo molecules. In any case, the observed change in equilibrium constant was in the right direction and of the right order of magnitude. Femtosecond measurements have recently shown that the energy transfer equilibrium between P680* and the accessory Chl is approximately 1:1 prior to charge separation [26]. This supports the view that it is possible to make a rough estimate of the equilibria in reaction centres by modelling the system as a Boltzmann distribution.

Throughout our studies we have noted that the RC IIb form of the complex was slightly more susceptible to photoinduced inactivation than RC IIa. The higher sensitivity to photodamage of RC IIb compared to RC IIa is consistent with recent findings that one of the β-carotene molecules and some Chl within the RC IIa type reaction centre are easily photooxidized by $P680^+$ and irreversibly bleached [27]. These secondary oxidations probably contribute to the partial protection of RC IIa against photoinduced inactivation [28].

Thus, the RC IIb activity in secondary electron transport from a donor to an artificial acceptor was about 84% of that of RC IIa. This is reasonable, if we consider that the RC IIb has been treated with Triton X-100 for a longer period of time in the chromatography column. Also, the loss of pigments could affect the stability of the RC IIb, and could be particularly true when one β-carotene is removed from the complex [27].

It seems that the non-essential chromophores for the PS II reaction centre photochemistry correspond to those which are stripped off the protein matrix during the isolation of the RC IIb complex and could be those that have no counterpart in the bacterial reaction centre. A basic question has emerged about the involvement of these extra chromophores in the PS II reaction centre based on analogies with the purple bacterial system. Whether their only function is to stabilize and to protect against photoinduced inactivation has yet to be determined. It could also be that the additional chromophores are located to the outside region of the reaction centre complex where they may function to facilitate energy transfer from the antenna complexes, CP43 and CP47, to P680.

This paper shows that it is possible to obtain a modified PS II reaction centre with different pigment stoichiometry without significantly affecting the structure and function of this chromoprotein. The comparative study of more of these PS II preparations containing a different number of chromophores should be very valuable for further and deeper studies on the kinetics of energy trapping and primary charge separation within the PS II reaction centre.

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