ELSEVIER

Biochimica et Biophysica Acta 1231 (1995) 21–28

BBA Biochimica et Biophysica Acta

Determination of the pigment stoichiometry of the photochemical reaction center of Photosystem II

Camiel Eijckelhoff *, Jan P. Dekker

Department of Physics and Astronomy, Institute of Molecular Biological Sciences, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands

Received 17 November 1994; revised 21 March 1995; accepted 31 March 1995

Abstract

The stoichiometry of chlorophyll *a*, pheophytin *a* and β-carotene in the photochemical reaction center of Photosystem II was analyzed by reversed phase high-performance liquid chromatography (HPLC) with methanol as the mobile phase, and by the shape of spectra of extracts in 80% acetone. For the HPLC method the molar extinction coefficient of pheophytin *a* in methanol was redetermined, while for the spectroscopic method spectra of extracts in 80% acetone were simulated by fitting with spectra of isolated chlorophyll *a*, pheophytin *a* and β-carotene in 80% acetone. Both methods give internally consistent results, and suggest that the reaction center of Photosystem II isolated by a short Triton X-100 treatment binds 6 chlorophyll *a* per 2 pheophytin *a* molecules. We also present evidence that prolonged exposure of the Photosystem II reaction center complex to Triton X-100 does not result in the loss of chlorophyll from the complex. Based on a comparison with spectra reported in publications from other groups, we conclude that the chlorophyll to pheophytin ratio has previously been underestimated to sometimes very significant extents, and that, as yet, no Photosystem II reaction center particles have been purified that bind less than 5–6 chlorophyll *a* per 2 pheophytin *a*.

Keywords: Photosystem II; Reaction center; Chlorophyll; Pheophytin; HPLC

1. Introduction

Since the isolation and purification by Nanba and Satoh [1] of the photochemical reaction center of PS II, our knowledge about the primary energy transfer and charge separation reactions within the reaction center has increased significantly (see [2,3] for recent reviews). The D1, D2, cytochrome *b*-559 and psb-I proteins together constitute the framework for binding a structure of chlorophyll *a*, pheophytin *a* and β-carotene molecules which are responsible for the ultrafast electron transfer from a Chl *a* species called P680 to a nearby Pheo *a* molecule. The sequence of electron transfer reactions in PS II undoubtedly resembles those in the well-resolved reaction center of photosynthetic purple bacteria, which is of the same type as the reaction center of PS II [4]. Important differences are found in the ability of PS II to use water as the ultimate electron donor, and in the overall spectroscopy, with well-resolved absorption bands in the bacterial RC and strongly overlapping absorption bands in the PS II RC [2,3].

Despite substantial interest in the structure and function of the isolated PS II RC, its pigment composition is still a matter of considerable debate. A number of reports have suggested that the PS II RC binds 6 Chl *a*, 2 Pheo *a* and 2 β-Car molecules [5,6], implying that the reaction center of PS II binds 2 chlorophylls and 1 carotenoid more than the reaction center of purple bacteria. The presence of the extra β-Car molecule was not observed in all preparations [7]. More recently, it was reported that prolonged incubation with the detergent Triton X-100 leads to the removal of the extra Chl *a* and β-Car molecules from the complex [8], and thus that it would be possible to purify a PS II RC complex with the same composition as the bacterial RC complex. This result led these authors to suggest that the extra pigments are located near the exterior of the D1-D2-cyt.*b*-559 complex. The preparation method of the com-

Abbreviations: Car, carotene; Chl, chlorophyll; HPLC, high-performance liquid chromatography; Pheo, pheophytin; PS, Photosystem; RC, reaction center.

* Corresponding author. Fax: +31 20 4447899; e-mail: camiel@nat.vu.nl.

SSDI 0005-2728(95)00055-0

plex with a 4:2:1 ratio of Chl *a*:Pheo *a*:β-Car has been improved [9], and it was suggested [10,11] that the extra pigments may not arise from the reaction center itself but from a contamination with CP47 or other Chl *a*-containing antenna complexes, and that, in analogy with the purple bacterial reaction center, the purified reaction center of PS II indeed would contain 4 Chl *a*, 2 Pheo *a* and 1 β-Car molecules.

One source of confusion regarding the pigment stoichiometry of the PS II RC may have arisen from the very different procedures that have been used in several laboratories to determine the stoichiometries, i.e., a number of HPLC methods [5–7] and spectroscopic methods [8–11]. In this report, we analyze these procedures in detail, and also address the question to which extent prolonged incubation with Triton X-100 leads to the extraction of pigments. We present simple HPLC and spectroscopic methods in two different solvents (100% methanol and 80% acetone) to determine the Chl *a*/Pheo *a*/β-Car ratio and stress the importance of the exact knowledge of the molar extinction coefficients of the pigments in the applied solvents. Based on a comparison with spectra reported in key publications by other groups, we conclude that the Chl/Pheo ratio has previously been underestimated by small [5,6] or large [8–11] extents, and that the isolation methods of the PS II RC based on incubation with Triton X-100 give rise to one type of particle binding 6 Chl *a* and 2 Pheo *a*.

2. Materials and Methods

2.1. Preparation of PS II RC complexes

PS II RC (D1-D2-cyt.b559) complexes were isolated from spinach CP47-RC complexes [12] as described before by Kwa et al. [13]. In short, CP47-RC at a concentration of about 1 mM Chl was diluted 10-fold with a solution of 2.5% (w/v) Triton X-100 in BTT (20 mM BisTris, 20 mM NaCl, 10 mM $MgCl_2$ and 1.5% taurine at pH 6.5), incubated for 15 min (4°C, dark), after which ion-exchange chromatography was used to separate the PS II RC from Triton-denatured CP47 and to exchange Triton X-100 to 0.03% n-dodecyl β,D-maltoside. Preparations obtained by this procedure have previously been characterized by a large number of spectroscopic techniques (see, e.g., [13–16]), and are from here on referred to as RC_{short}, the suffix indicating the relatively short time of exposure to the detergent Triton X-100.

In order to study the influence of the time of Triton exposure, we also prepared particles as described above, but in which the washing with 0.2% Triton X-100 in BTT just after the sample was applied to the ion-exchange column was prolonged from about 15 min to 15 h. The PS II RC complexes obtained by this procedure are denoted RC_{long}.

2.2. Pigment extraction

For pigment analysis, the pigments were extracted with 80% acetone at room temperature under dim light conditions, sonicated for 1 min and centrifuged for 3 min to remove insoluble material. Alternatively, dilute samples were loaded on an extraction column (SECO Cartridge C18, 3 ml, pre-equilibrated with distilled water) and rinsed with water, after which the column was dried by gently flushing with nitrogen gas. For extraction, 1 ml of 100% acetone (Rathburn, HPLC-grade) was slowly applied to the column. It was checked that complete extraction occurred with both methods by repeating these procedures and by checking that no pigments (except cytochrome *b*-559) remained bound to the denatured protein mass.

2.3. HPLC analysis of pigment composition

Pigment analysis was performed using an HPLC-setup consisting of an HPLC-pump (Waters 600E) and a reversed phase column (Spherisorb C8-5, 250 × 4.6 mm) fitted with a guard cartridge (10 × 4 mm) containing the same material, and a Valco injection valve with a 200 μl sample loop. For detection a diode array detector (Waters 990) was used in the wavelength range of 350 to 650 nm with 2 nm resolution (with our detector it was not possible to measure at 650–700 nm). The system was equilibrated at room temperature with 100% methanol (Rathburn, HPLC-grade) as a mobile phase, running at a flow rate of 0.7 ml/min with a typical pressure of 1000 psi. Note that this method is sufficient to separate Chl *a*, β-Car and Pheo

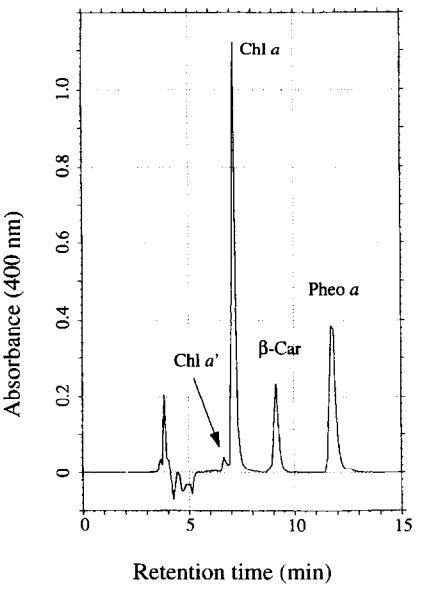

Fig. 1. HPLC-Chromatogram measured at 400 nm as described in Section 2.3 showing the separated pigments. Fractions containing Chl *a'*, Chl *a*, β-Car and Pheo *a* eluted at 6.6, 7.2, 9.1 and 11.8 min, respectively. The peaks observed at 3.5–5.5 min are due to the mixing of the injected solvent and the mobile phase.

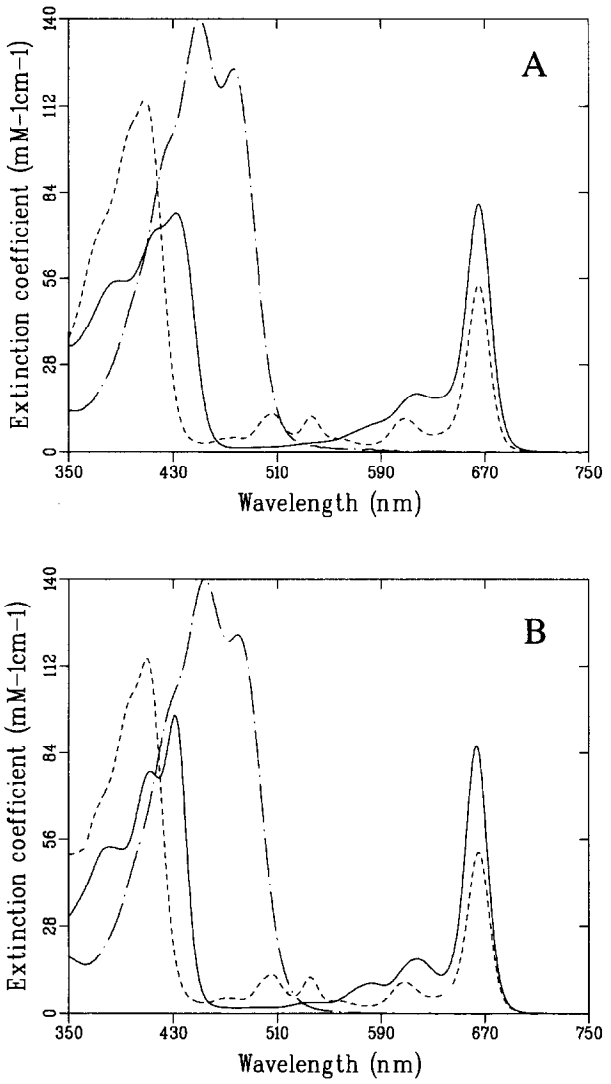

Fig. 2. Room-temperature absorption spectra of HPLC-purified Chl *a* (solid line), Pheo *a* (dashed line) and β-Car (dash-dotted line) dissolved in 100% methanol (A) or in 80% acetone (B).

a within 15 min (see also Fig. 1) and is easily reproduced because only one solvent is used. It was checked that 100% methanol does not induce pheophytinization of Chl *a* by reinjecting the Chl *a* fraction in the HPLC system; the only effect that could be observed was a small increase (of about 5%) of the Chl *a'* peak (note that rinsing with water does induce pheophytinization –see below). Peak areas of Chl *a* (including Chl *a'*), β-Car and Pheo *a* were determined at 618, 450 and 608 nm, respectively, with the Waters 990 measuring program.

For calculating the concentration of Chl *a* in 100% methanol, an extinction coefficient was used of 18.68 mM^{-1} cm^{-1} at 618 nm [17]. This corresponds, according to the absorption spectrum recorded on a Cary 219 spectrophotometer (Fig. 2A, full line), to values of 79.15 mM^{-1} cm^{-1} at 665 nm and 76.94 mM^{-1} cm^{-1} at 432 nm, which corresponds very well with the values reported in [17]. For calculating the concentration of β-Car in 100% methanol an extinction coefficient was used of 140 mM^{-1} cm^{-1} at 450 nm [17].

2.4. Determination of pheophytin *a* extinction coefficients

Because errors in the Pheo *a* extinction coefficients will have large effects on the calculated Chl/Pheo ratios, and because the Pheo *a* spectra in 100% methanol in our hands (Fig. 2A, dashed line) and in [17] showed somewhat different peak maxima, we redetermined the extinction coefficient of Pheo *a* in 100% methanol. For this we made use of the extraction column described above for the concentration of dilute samples. However, instead of intact particles we now loaded purified Chl *a* in methanol on the column and made use of the fact that the bound Chl (which is now exposed because of the absence of the protein shell) partly pheophytinizes when it is rinsed with water. In principle, complete pheophytinization may be achieved by acidification; however, this procedure was avoided because acidified material gave rise to a variety of peaks in the HPLC chromatogram, while the water-induced Pheo *a* yielded a single peak in the chromatogram at the same position as the Pheo *a* of the PS II RC extract.

A concentrated solution of Chl *a* in 100% methanol, purified by chromatography over the Spherisorb C-8 column as described above, was divided into two portions of 100 μl. One portion was diluted to a total volume of 3.0 ml in order to serve as a standard solution. The other portion was loaded on the water-equilibrated extraction column described above. The Chl *a* was partly (20–25%) pheophytinized by rinsing with distilled water, after which the column was blown dry and the mixture was eluted and diluted with methanol to give a 3.0 ml solution. Identical aliquots of both portions were injected into the HPLC-system, and yielded single peaks in the chromatogram with retention times of 7.2 min for Chl *a* and 11.8 min for Pheo *a*. After comparison of the spectra, and assuming an extinction coefficient of 18.68 mM^{-1} cm^{-1} at 618 nm for Chl *a* (see above), values of 10.68 mM^{-1} cm^{-1} at 608 nm, 114.3 mM^{-1} cm^{-1} at 409 nm and 53.97 mM^{-1} cm^{-1} at 665 nm for Pheo *a* were estimated (see also Fig. 2A, dashed line). These values are about 10% higher than reported by Lichtenthaler [17] for Pheo *a* in 100% methanol, which may be related to the slightly different peak positions of the absorption bands of Pheo *a* in methanol in Lichtenthaler's and our spectra.

2.5. Spectroscopic analysis of pigment composition

The pigment stoichiometry was also analyzed by spectroscopic methods, including those introduced and applied by Picorel and co-workers [8–11]. A spectrum was recorded of an extract in 80% acetone and the amplitude of the Pheo Q_x absorption band was determined at 535 nm. Obviously, some broad baseline subtraction is required, which is not

specified in [8–11]. Here, we took the difference between the absorbances at 535 nm (the peak of the Pheo Q_x absorption band) and 551 nm (the absorbance minimum between the Pheo Q_x and Chl Q_x absorption bands). The amplitude of the Pheo Q_x absorption band was compared with that of the Chl/Pheo $Q_{y(0\ 0)}$ absorption band at 663 nm. In [8–11], the extract was subsequently pheophytinized by 15 mM HCl and again a spectrum was recorded, from which the Q_y absorbance at 665 nm was determined. In those studies, the Chl/Pheo ratio was determined by using a value of 49.3/6.504 = 7.58 for the Q_y and Q_x absorbances of the pheophytinized extracts.

We extended the analyses in [8–11] by simulating 80% acetone spectra of well-defined composition. For this, we collected the Chl *a*, Pheo *a* and β-Car fractions in 100% methanol from HPLC experiments as described above, dried the fractions by flushing with nitrogen gas, resolubilized the fractions in 80% acetone or in 100% acetone, and recorded their absorption spectra on a Cary 219 spectrophotometer. In Fig. 2B the spectra of Chl *a*, Pheo *a* and β-Car in 80% acetone are shown after normalization according to the extinction coefficients in 80% acetone (86.3 mM^{-1} cm^{-1} at 663 nm for Chl *a*, 51.9 mM^{-1} cm^{-1} at 665 nm for Pheo *a* and 140 mM^{-1} cm^{-1} at 454 nm for β-Car –values from Ref. [17]). For comparison, the spectra in 100% methanol are shown as well (Fig. 2A). The spectra (in 80% acetone or in 100% acetone) were summed in a number of fixed stoichiometries, and the absorbances of the Pheo Q_x bands (535–551 nm, see above) and the Chl/Pheo Q_y bands (663 nm) of the simulated spectra were recorded, as well the absorbances of the Soret bands at 412 and 431 nm (80% acetone) or 415 and 427 nm (100% acetone).

3. Results

3.1. HPLC analysis

The standard preparation which serves as a reference for all methods described in this paper is RC_{short}, the PS II RC complex that is obtained by a short incubation with Triton X-100 of the CP47-RC complex of PS II. This method was applied for the first time in [12] and optimized in [13]. The method is basically similar to the one reported by van Leeuwen et al. [7], and yields particles with virtually identical 4 K absorption spectra [15,18]. SDS-PAGE has revealed that these particles are essentially free of CP47 [7,12], and based on Chl yields and HPLC results, they were estimated to contain 6 Chl *a* and 2 Pheo *a* per complex [7].

In order to establish to which extent the time of exposure to Triton X-100 influences the pigment composition of the PS II RC, we isolated RC_{short} and RC_{long} with procedures as described in Section 2.1 (which only differ in an about 30-fold difference in time of exposure to Triton), and determined the pigment stoichiometries by HPLC analysis. The final contents were 6.40 ± 0.25 Chl *a* and 1.58 ± 0.26 β-Car per 2.00 Pheo *a* for RC_{short} and 6.31 ± 0.28 Chl *a* and 1.34 ± 0.23 β-Car per 2.00 Pheo *a* for RC_{long} (average of 8 and 6 different preparations, respectively). The values would change into 5.92 and 5.84 Chl *a* per 2.00 Pheo *a* if Lichtenthaler's extinction coefficients for Pheo *a* [17] had been applied. These results indicate that (under our particular conditions) Chl is not extracted from the complex by prolonged incubation with Triton (in contrast to the results in [8], but in agreement with the results presented in [19]), and suggest a stoichiometry of 6 Chl *a* per 2 Pheo *a* of the PS II RC.

3.2. Comparison of spectra of acetone extracts

Fig. 3A presents the spectrum of RC_{short} in 80% acetone (full line), together with simulated spectra in which the spectra of purified Chl *a*, Pheo *a* and β-Car in 80% acetone were normalized according to their extinction coefficients [17] and summed in a ratio of 6:2:2 (dashed line) or 4:2:1 (dash-dotted line). The only difference between the spectra of the extract and of the 6:2:2 simulation seems to be a slightly lower β-Car contribution in the extract, in agreement with the HPLC results described above. Note that the absorbances at 412 and 430 nm have about equal magnitudes in the extract and in the 6:2:2 simulation, whereas the ratio is about 1.15 in the 4:2:1 simulation.

The question arises to what extent our preparations with 6 Chl *a* per Pheo *a* compare to those with 4, 5 and 6 Chl *a* per 2 Pheo *a* reported by Picorel and co-workers [8–11] and to those with 6 Chl *a* per 2 Pheo *a* reported by other groups, i.e., those of Satoh [5] and Barber [6]. A straightforward way to address this issue is to compare the spectra of the acetone extracts. Yruela et al. [9] reported acetone spectra between 500 and 700 nm of their preparations characterized by 4:2 and 6:2 ratios of Chl:Pheo. In [8,10,11] acetone spectra of the preparations characterized by a 4:2 ratio of Chl:Pheo are not reported, due to which a direct comparison with our preparations is not possible, but because the pigment quantitation methods are presumably the same in [8–11], we may regard Ref. [9] as representative.

Table 1 gives the ratio of the amplitudes of the Chl/Pheo Q_y absorption band at 663 nm and the Pheo Q_x absorption band at 535 nm for our simulated spectra, some of our extracts and the data reported in [9]. The results with the simulated spectra suggest that this ratio depends not only on the Chl content, but also on the particular β-Car content. This can be explained by the tail of the β-Car absorption band, which contributes to a small extent to the absorption at 535 nm (see also Fig. 2B, dash-dotted line). The values for RC_{short} and RC_{long} (36 and 37, respectively) are somewhat higher than expected from 6:2:1.6 and 6:2:1.3 simulations (34.6 and 35.4, respectively). A best linear fit of the spectrum of the extract in

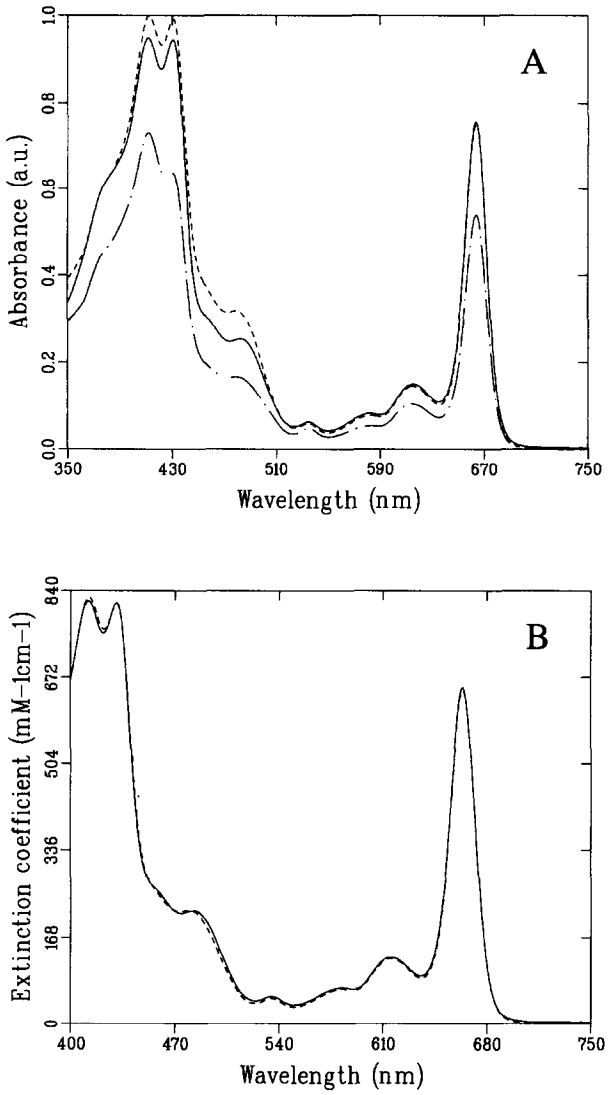

Fig. 3. (A) Room-temperature absorption spectra in 80% acetone of the pigment extract of RC_{short} (solid line), and of isolated Chl *a*, Pheo *a* and β-Car in 80% acetone summed according to their extinction coefficients [17] in ratios of 6:2:2 (dashed line) or 4:2:1 (dash-dotted line). (B) Best linear fit (dashed line) of the spectrum of RC_{short} in Fig. 3A (solid line) by addition of the spectra of isolated Chl *a*, Pheo *a* and β-Car in 80% acetone in a ratio of 6.31:2.00:1.63.

Fig. 3A with the constituent spectra was found with 6.31 Chl *a* and 1.63 β-Car per 2.00 Pheo *a* (Fig. 3B), which is in very good agreement with the HPLC results (Table 1). This indicates that the sets of extinction coefficients for the pigments in methanol (as used for the HPLC analysis) and 80% acetone (as used for the spectroscopic analysis) yield essentially the same results.

The surprising result with the spectra of the extracts is that the '4:2:1' preparation of Yruela et al. [9] yields an even higher ratio (41) than RC_{short} and RC_{long} (36–37), whereas the '6:2:2' preparation of Yruela et al. gives a rather extreme ratio. We conclude from these results that the '4:2:1' preparation of Yruela et al. [9] contains more than 6 Chl *a* per 2 Pheo *a* (a ratio of 41 is found for the 7:2:1 simulation), and that the '6:2:2' preparation of Yruela et al. contains more than 10 Chl *a* per 2 Pheo *a*. We thus may suspect that also the preparations in [8,10,11] with a supposed stoichiometry of 4 Chl *a* per 2 Pheo *a* contain considerably more than 4 Chl *a* per 2 Pheo *a*.

In [5,6] complete spectra are shown of acetone extracts of preparations characterized by a 6 Chl *a* per 2 Pheo *a*. In this case, it is more convenient to analyze the ratio of absorbances at 412 and 431 nm. Table 2 gives this ratio for our simulated spectra, some of our extracts and the data reported in [5,6]. The results with the simulated spectra indicate that this ratio, too, is influenced by the particular β-Car content, which is caused by a larger contribution of the β-Car spectrum at 431 nm than at 412 nm (Fig. 2B, dash-dotted line). The values for RC_{short} and RC_{long} (1.01 and 1.03, respectively) are somewhat lower than expected from 6:2:1.6 and 6:2:1.3 simulations (1.03 and 1.04, respectively), which indicates a slightly higher Chl/Pheo

Table 1
Ratios of the amplitudes in 80% acetone spectra of the Chl/Pheo Q_y absorption band at 665 nm and the Pheo Q_x absorption band at 535 nm, measured as described in section 2 as the difference in absorption at 535 and 551 nm

Preparation	$A_{665}/(A_{535}-A_{551})$	Chl:Pheo:β-Car ratio
6:2:2 simulation	33.6	6.0:2.0:2.0
6:2:1 simulation	36.2	6.0:2.0:1.0
5:2:1 simulation	31.4	5.0:2.0:1.0
4:2:1 simulation	26.5	4.0:2.0:1.0
RC_{short}	36	6.4:2.0:1.6
RC_{long}	37	6.3:2.0:1.3
'4:2:1' - Yruela	41	
RC(1.05)	50	9.6:2.0:2.0
'6:2:2' - Yruela	66	

The Chl:Pheo:β-Car ratio is based on HPLC results as described in section 2.3. See the legend of Fig. 3 for details of the simulated spectra. RC(1.05) reflects a preparation with a significant CP47 contamination, characterized by a ratio of the room temperature absorbances at 416 and 435 nm of 1.05. The data of the '6:2:2'/'4:2:1'-Yruela preparations were calculated from Fig. 1 of Ref. [9].

Table 2
Ratios of the amplitudes in 80% acetone spectra of the Soret absorption bands at 412 and 431 nm

Preparation	A_{412}/A_{431}	Chl:Pheo:β-Car ratio
6:2:2 simulation	1.01	6.0:2.0:2.0
6:2:1 simulation	1.06	6.0:2.0:1.0
5:2:1 simulation	1.10	5.0:2.0:1.0
4:2:1 simulation	1.15	4.0:2.0:1.0
RC_{long}	1.03	6.3:2.0:1.3
RC_{short}	1.01	6.4:2.0:1.6
'6:2:2'-Kobayashi	0.93	
'6:2:2'-Gounaris	0.94	

The Chl:Pheo:β-Car ratio is based on HPLC results as described in section 2.3. See the legend of Fig. 3 for details of the simulated spectra. The data of the '6:2:2'-Kobayashi and '6:2:2'-Gounaris preparations were calculated from the spectra in Ref. [5] and Ref. [6], respectively.

ratio than 6/2, in agreement with the results from the HPLC analysis and those from the analysis in Table 1.

Table 2 also shows that the extracts in Refs. [5] and [6] are characterized by a significantly lower A_{412}/A_{431} ratio (~ 0.94) than of the 6:2:2 simulation (1.01), which suggests that these preparations contain more than 6 Chl per 2 Pheo. Some caution is justified here, however, because in both studies supposedly 100% acetone was used. We also performed the simulations with spectra of purified pigments in 100% acetone, and found an A_{415}/A_{427} ratio of 0.96 for the 6:2:2 simulation (not shown). The different ratio may be related to the different peak positions of the Soret bands of Pheo and Chl, which we found to be separated by 12 nm in 100% acetone and 19 nm in 80% acetone. Similar separations of Pheo and Chl Soret bands in 100% and 80% acetone were reported in [17]. Because in [5,6] the peak positions were separated by 19 nm, we suspect the presence of some water in these extracts, due to which the simulations in 80% acetone may give the most reliable comparison. We conclude from this analysis that the preparations described in Refs. [5,6], which are frequently cited as standard preparations for PS II RC particles binding 6 Chl *a* and 2 Pheo *a*, in fact contain at least slightly more than 6 Chl *a* per 2 Pheo *a*.

3.3. Comparison of spectra of isolated complexes

It is also possible to judge the composition of PS II RC complexes by their room temperature absorption spectra. In this case, the information may only be regarded as qualitative, because pigment-pigment and pigment-protein interactions will modify the intensities and energies of the absorption bands. The absorption properties of cytochrome *b*-559 will also influence the spectra.

The room temperature absorption spectra of RC_{short} and RC_{long} are presented in Fig. 4. The ratio of absorbances at 416 nm and 435 nm is 1.20 for RC_{short} and 1.27 for RC_{long}. These ratios are much higher than observed for the isolated pigments, which is at least in part explained by the absorption of cytochrome *b*-559 at 416 nm. The higher ratio for RC_{long} may be related to the lower β-Car content. For comparison, from the spectra of the '6:2:2' preparations in Refs. [6] and [19] ratios of about 1.15 can be calculated (somewhat lower than of RC_{short}, suggesting a slightly higher Chl content and/or a higher β-Car content), while the '6:2:1' preparation [19] yields a ratio of about 1.27 (similar to RC_{long}). The spectra of the preparations of Montoya et al. [20] (including those supposed to contain only 4 Chl per 2 Pheo) also yield ratios of about 1.15, while the main fraction in [10] (which is also supposed to contain 4 Chl per 2 Pheo) shows a ratio of about 1.20, which suggests in view of the very low β-Car content, even a higher Chl *a* content than RC_{long} and the preparation in [19].

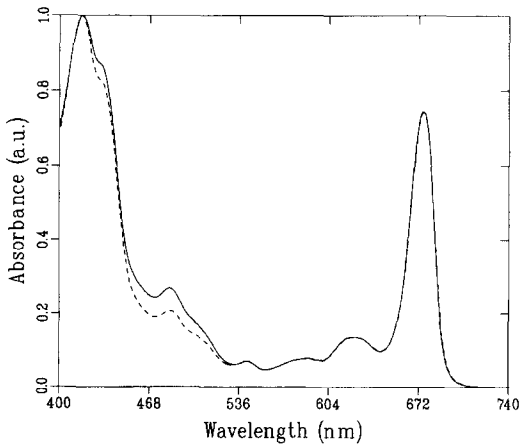

Fig. 4. Absorption spectra at room temperature of RC_{short} (solid line) and RC_{long} (dashed line).

We also calculated the ratio of the Chl/Pheo Q_y and Pheo Q_x absorption bands. The ratios observed for RC_{short} and RC_{long} were 32 ± 3. We observed a larger variation in this ratio than in the corresponding ratio of the acetone extracts. The general tendency, however, is that this ratio is somewhat lower in the intact complexes (about 32) than in the isolated pigments in 80% acetone (about 36 –see Table 1), which may be explained by the energetic heterogeneity of the Q_y transitions of the chlorophylls in the intact complexes (the 670 and 680 nm absorbing species). The only spectrum of a supposed 4:2 preparation from which this ratio could be estimated [21] yielded a value of about 31–35, which is about the same as observed for RC_{short} and RC_{long}.

The main conclusion from this qualitative comparison of room temperature absorption spectra is that the preparations of Montoya et al. [20,21] with a supposed 4:2:1 stoichiometry do not contain significantly less Chl per Pheo than our preparations, which supports the results of the comparative analysis of the spectra of the pigments in acetone (section 3.2), and suggests that the minimal Chl content of the PS II RC preparations isolated thus far is 6 Chl *a* per 2 Pheo *a*.

4. Discussion

In this report two methods are described to analyze the Chl *a*/Pheo *a*/β-Car stoichiometry of the PS II RC, i.e., an HPLC analysis of the isolated pigments in 100% methanol and a spectroscopic analysis of the isolated pigments in 80% acetone. The spectroscopic method is entirely based on the extinction coefficients of the pigments in acetone reported by Lichtenthaler [17], whereas for the HPLC experiments the Pheo *a* extinction coefficient in 100% methanol had to be redetermined. By using the new values, results were obtained that were consistent with those based on the extinction coefficients in 80% acetone. Combined with the comparison of spectra from other reports, our results suggest that, as yet, no PS II RC

preparations have been purified with a lower Chl/Pheo ratio than 5–6/2.

The pigment quantitation methods explored in this report suggest that most of the previous estimates of the Chl/Pheo stoichiometry result in a small [5,6] or a severe [8–11,20,21] underestimation of the Chl/Pheo ratio. Because in most of these studies (especially those in [8–11,20,21]) essential details of the pigment quantitation methods are missing, we can only speculate on the reason of the discrepancies. The HPLC analyses in [5,6] were performed in mixtures of solvents, and because of the large influence of the particular solvent on the spectra of the pigments (see, e.g., Fig. 2A and B), the extinction coefficients should be determined for each of these mixtures. It is not clear whether or not this is done. This knowledge is important, because if, for instance, extinction coefficients for 80% acetone are used for chlorophylls and pheophytins in methanol, the Chl/Pheo ratio is underestimated by about 15%. We also can only speculate on the more severe underestimation in the quantitation methods presented by Picorel and co-workers. One gets the impression that the total absorption at 535 nm is recorded and fully attributed to the Pheo Q_x absorption band [8]; however, from Fig. 2B it becomes clear that also Chl and β-Car significantly contribute to the absorption at this wavelength.

The results described in this report support the view that prolonged incubation with Triton X-100 does not result in the extraction of Chl *a* from the PS II RC complex and that the preparation methods based on incubation with Triton X-100, including those reported in [8–11], yield PS II RC particles that bind 6 Chl *a* and 2 Pheo *a*. This means that these procedures give rise to one type of particle, which, if prepared properly, is pure and not contaminated with other pigment-protein complexes or uncoupled pigments. The results also justify the approach to use the well-resolved CP47-RC or PS II core complex as starting material [7,13], because in this case only a very short incubation period of Triton X-100 is required, due to which PS II RC particles can easily be obtained with high purity, reproducibility and yield. A consequence of our conclusion is that preparations with a higher Chl:Pheo ratio than 6:2 (or 4:2 in [8–11]) are contaminated by other pigment-protein complexes. We note in this respect that it can not be excluded that the slightly higher ratio than 6:2 found for our preparations could be due to a minor contamination and/or a heterogeneity in our preparations. In [11] it was shown by high-resolution spectroscopy that CP47 is largely responsible for such contaminations.

There is now considerable evidence that the PS II RC binds Chl and Pheo in similar, but not identical ways as the purple bacterial RC [2,3,22]. This includes the Chl on which the triplet of P680 is localized and which has the same orientation as the accessory BChl in the bacterial RC [23–25]. Thus, it seems not unreasonable to assume that the PS II RC contains a structure of 4 Chl *a* and 2 Pheo *a* that resembles the structure of the purple bacterial RC, but in which by some way the interaction between the 'special pair' molecules is diminished [22,26]. However, there is also substantial evidence that the PS II RC complex binds one or two accessory Chl molecules that are responsible for reduction of $P680^+$ at cryogenic temperatures [27] and slow (20–30 ps) energy transfer to P680 [28]. The histidines 118 of the D1 and D2 proteins have been suggested to bind these accessory Chl molecules [5,27,28].

Acknowledgements

We thank Drs. R. van Grondelle, H.J. van Gorkom and A.R. Holzwarth for stimulating discussions. This research was supported in part by the Dutch Foundation for Chemical Research (SON) financed by the Netherlands Organisation for Scientific Research (NWO), and by the EC, grant CT940619.

References

[1] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[2] Van Grondelle, R., Dekker, J.P., Gillbro, T. and Sundström, V. (1994) Biochim. Biophys. Acta 1187, 1–65.
[3] Diner, B.A. and Babcock, G.T. (1995) In Oxygenic Photosynthesis: The Light Reactions (Yocum, C.F. and Ort, D., eds.), Kluwer, Dordrecht, in press.
[4] Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
[5] Kobayashi, M., Maeda, H., Watanabe, T., Nakane, H. and Satoh, K. (1990) FEBS Lett. 260, 138–140.
[6] Gounaris, K., Chapman, D.J., Booth, P., Crystall, B., Giorgi, L.B., Klug, D.R., Porter, G. and Barber, J. (1990) FEBS Lett. 265, 88–92.
[7] Van Leeuwen, P.J., Nieveen, M.C., Van de Meent, E.J., Dekker, J.P. and Van Gorkom, H.J. (1991) Photosynth. Res. 28, 149–153.
[8] Montoya, G., Yruela, I. and Picorel, R. (1991) FEBS lett. 283, 255–258.
[9] Yruela, I., Van Kan, P.J.M., Müller, M.G. and Holzwarth, A.R. (1994) FEBS Lett. 339, 25–30.
[10] Aured, M., Moliner, E., Alfonso, M., Yruela, I., Toon, S.P., Seibert, M. and Picorel, R. (1994) Biochim. Biophys. Acta 1187, 187–190.
[11] Chang, H.-C., Jankowiak, R., Reddy, N.R.S., Yocum, C.F., Picorel, R., Seibert, M. and Small, G.J. (1994) J. Phys. Chem. 98, 7725–7735.
[12] Dekker, J.P., Bowlby, N.R. and Yocum, C.F. (1989) FEBS Lett. 254, 150–154.
[13] Kwa, S.L.S., Newell, W.R., Van Grondelle, R. and Dekker, J.P. (1992) Biochim. Biophys. Acta 1099, 193–202.
[14] Groot, M.-L., Peterman, E.J.G., Van Kan, P.J.M., Van Stokkum, I.H.M., Dekker, J.P. and Van Grondelle, R. (1994) Biophys. J. 67, 318–330.
[15] Kwa, S.L.S., Eijckelhoff, C., Van Grondelle, R. and Dekker, J.P. (1994) J. Phys. Chem. 98, 7702–7711.
[16] Roelofs, T.A., Kwa, S.L.S., Van Grondelle, R., Dekker, J.P. and Holzwarth, A.R. (1993) Biochim. Biophys. Acta 1143, 147–157.
[17] Lichtenthaler, H.K. (1987) Methods Enzymol. 148, 350–382.
[18] Van Leeuwen, P.J. (1993) Doctoral Thesis, University of Leiden, The Netherlands.
[19] De Las Rivas, J., Telfer, A. and Barber, J. (1993) Biochim. Biophys. Acta 1142, 155–164.
[20] Montoya, G., Cases, R., Yruela, I. and Picorel, R. (1993) Photochem. Photobiol. 58, 724–729.

[21] Montoya, G., Cases, R., Yruela, I. and Picorel, R. (1992) In Research in Photosynthesis (Murata, N., ed.), Vol. II, pp. 73–76, Kluwer Academic Publishers, The Netherlands.
[22] Durrant, J.R., Klug, D.R., Kwa, S.L.S., Van Grondelle, R., Porter, G. and Dekker, J.P. (1995) Proc. Natl. Acad. Sci. USA, in press.
[23] Van Mieghem, F.J.E., Satoh, K. and Rutherford, A.W. (1991) Biochim. Biophys. Acta 1058, 379–385.
[24] Van der Vos, R., Van Leeuwen, P.J., Braun, P. and Hoff, A.J. (1992) Biochim. Biophys. Acta 1140, 184–198.
[25] Kwa, S.L.S. (1993) Doctoral Thesis, Vrije Universiteit, Amsterdam.
[26] Braun, P., Greenberg, B.M. and Scherz, A. (1990) Biochemistry 29, 10376–10387.
[27] Koulougliotis, D., Innes, J.B. and Brudvig, G.W. (1994) Biochemistry 33, 11814–11822.
[28] Schelvis, J.P.M., Van Noort, P.I., Aartsma, T.J. and Van Gorkom, H.J. (1994) Biochim. Biophys. Acta 1184, 242–250.