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Stoichiometry of cytochrome *b*-559 in Photosystem II

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A careful determination of the stoichiometry of cytochrome *b*-559 per Photosystem II reaction centre has been made for different Photosystem II preparations. Using an extinction coefficient determined from the α-band of reduced pyridine haemochrome and chemical analyses of pigment levels it is concluded that there is one cytochrome *b*-559 haem per Photosystem II reaction centre. This conclusion assumes that by analogy with the purple bacterial reaction centre there are two pheophytin molecules per Photosystem II reaction centre.

Introduction

For some years it has been clear that high potential cytochrome *b*-559 is a component of Photosystem II (PS II) [1]. Indeed under conditions when water oxidation is inhibited this cytochrome can donate electrons to the photo-oxidised reaction centre chlorophyll P-680 [2]. This electron donation can occur at very low temperatures indicating that the cytochrome is closely located to the reaction centre [3]. Confirmation of this intimate relationship has come from the isolation of various PS II protein complexes [4] including a reaction centre complex composed of the D1 and D2 polypeptides [5,6]. Cytochrome *b*-559 seems to be composed of two subunits, α and β, which bind one haem [7] and which are encoded for in higher plants by two chloroplast genes known as *psb*E and *psb*F, respectively [8,9]. Despite many speculations (see Refs. 7 and 10) it is not known what the precise function of the cytochrome is and there are doubts regarding its stoichiometry per PS II reaction centre. Several reports suggest that there are two haems within a PS II complex (e.g., Refs. 11 and 12) while others have argued the case for one (e.g., Refs. 13 and 14). In order to fully elucidate the role of this cytochrome it is vital to solve this controversy. For this reason we have set out to determine carefully the cytochrome *b*-559 content of various types of PS II particles.

Materials and Methods

Isolation procedures

Oxygen evolving PS II particles (K-M particles) were prepared from spinach chloroplasts by the method of Kuwabara and Murata [15] and suspended in buffer solution A (0.2 M sucrose, 20 mM NaCl, 20 mM Mops (pH 7.0)). When stored in liquid N_2, 70% by volume of glycerol was also added to the suspension. Removal of light-harvesting chlorophyll proteins (LHC II) from the K-M particles was performed by the method of Ghanotakis et al. [16] with a slight modification. Briefly the K-M particles were suspended in solution B (0.4 M sucrose, 50 mM Mes (pH 6.0), 10 mM NaCl) at a chlorophyll (Chl) concentration of 2.5 mg/ml. This was mixed with an equal volume of solution C (1.0 M sucrose, 50 mM Mes (pH 6.0), 10 mM $CaCl_2$, 0.8 M NaCl, 70 mM *n*-octyl-β-D-glucoside (OG)) and incubated for 10, 30, 60 or 90 min in the dark at 4°C. After incubation for given periods, each solution was diluted with twice the volume of solution D (1 M sucrose, 50 mM Mes (pH 6.0), 5 mM $CaCl_2$, 350 mM NaCl, 5 mM OG) and further incubated for 5 min. The solutions were centrifuged at 38000 × *g* at 4°C for 90 min and the resulting supernatant treated as described

Abbreviations: DEAE, diethylaminoethyl; HPLC, high performance liquid chromatography; OG, *n*-octyl-β-D-glucoside; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; PS II, Photosystem II; LHC II, light-harvesting chlorophyll protein; Chl, chlorophyll

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in Ref. 16 to obtain the Ghanotakis et al.-type PS II core complex.

In addition to the above preparations, a PS II reaction centre complex was prepared by the method of Nanba and Satoh [5] with some changes. The K-M particles were treated with 4% (vol.) Triton X-100, 50 mM Tris-HCl (pH 7.2) for 1 h on ice with stirring. The suspension was then centrifuged at $100000 \times g$ for 1 h at 4°C and the supernatant solution was divided into several aliquots to be subjected to separate DEAE-Toyopearl (obtained from Toyo Soda Co. Ltd., Tokyo, Japan) columns equilibrated with solution E (50 mM Tris-HCl (pH 7.2), 0.2% Triton X-100, 5 mM NaCl). Each column was then washed with the equilibration solution for 1, 2, 5, 12, or 24 h at 4°C in the dark. After washing for given periods, the eluent was changed to a solution of 0.05% Triton X-100, 50 mM Tris-HCl (pH 7.2), 5 mM NaCl and subsequently subjected to gradient elution of NaCl (5–200 mM). The elution profiles varied depending on the pre-washings and various fractions were collected. Nanba and Satoh reaction centre particles eluted at about 100 mM.

Cytochrome *b*-559 was purified from the PS II particles according to the procedure described by Metz et al. [17]. However, the final procedure to remove the majority of Triton X-100 and urea and to concentrate cytochrome *b*-559 was modified as follows. The fraction containing cytochrome *b*-559 eluted out from a DEAE-cellulose column (Wattman DE52) was loaded on a DEAE-Sephadex column (Pharmacia A-50), pre-equilibrated with a solution of 50 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol, 0.05% Triton X-100 and washed with the same solution. Cytochrome *b*-559 which bound to the DEAE-Sephadex was then eluted out from the column with a solution of 50 mM Tris-HCl (pH 8.0). 0.5 mM dithiothreitol, 200 mM NaCl. The fractions containing cytochrome *b*-559 were collected and immediately submitted to spectroscopic analysis of cytochrome *b*-559.

Pigment analysis

Pigment extraction from the PS II preparations was carried out according to the method of Watanabe and Kobayashi [18]. The PS II K-M particles, the Ghanotakis et al.-type PS II core and Nanba and Satoh-type PS II reaction centre complexes were sedimented by centrifugation at 4°C and the supernatant discarded. Each of the resulting pellets were ground in a mortar for 1 min with 25 g of anhydrous $NaHPO_4$ (−20°C) as desiccant. The ground material was transferred into a glass beaker to which 20 ml of chloroform (−20°C) was added and sonicated for 30 s. The supernatant solution was decanted into a glass centrifuge tube and the residue re-extracted twice with 10 ml of chloroform. The chloroform solutions were pooled and centrifuged at $26000 \times g$ for 5 min to remove $NaHPO_4$ powder. The supernatant was immediately subjected to rotary evaporation until dryness. The green dried material obtained was redissolved in 400 µl of absolute ethanol and a 250 µl aliquot was injected into an HPLC (Waters 6000A, reverse-phae column of µBond PAK C_{18}). Elution of pigments was done by methanol/ethanol (65 : 35, vol/vol) solution and monitored at 270 nm. The molar ratio of separated pheophytin *a* (Pheo *a*) to chlorophyll *a* (Chl *a*) and that of chlorophyll *b* (Chl *b*) to chlorophyll *a* were determined spectrophotometrically in 80% acetone using millimolar extinction coefficients of 49.3 at 666 nm for Pheo *a*, 47.6 at 648 nm for Chl *b* and 81.1 at 664 nm for Chl *a* [19]. Combining the molar ratios obtained and the absorbance of the corresponding sample at the peak, 664–666 nm in 80% acetone, the amount of Pheo *a* in the sample was determined.

Spectroscopic assay of cytochrome b-559

The concentration of cytochrome *b*-559 in PS II cores, reaction centres and K-M particles, as well as purified cytochrome *b*-559, was determined by employing two different methods. (1) Redox difference spectra of cytochrome *b*-559 were measured by using a dual-beam spectrophotometer equipped with memory function (Hitachi 320) as dithionite-reduced minus ferricyanide (FeCN)-oxidised spectra in the presence and absence of Triton X-100. (2) The haem derived from cytochrome *b*-559 was assayed by slight modification of the method of Appleby [20] as the reduced pyridine haemochrome. One volume of each sample solution was added to three volumes of solution containing 0.1 M NaOH, 0.267 mM $K_3Fe(CN)_6$, 33% vol. pyridine and immediately its absorption spectrum was measured and memorised. A few grains of sodium dithionite were then added to a cuvette containing the above solution and dithionite minus FeCN difference spectrum obtained.

Results

Extinction coefficients of cytochrome b-559

With the purpose of determining the number of cytochrome *b*-559 per PS reaction centre, we compared the levels of cytochrome in the different types of PS II preparations. As mentioned above, cytochrome *b*-559 almost certainly possesses one protohaem per α/β subunit as the prosthetic group. The protohaem concentration of *b*-type cytochromes is usually measured through the characteristic α-band absorption at 556 nm of the reduced pyridine haemochrome with a millimolar extinction coefficient of $\varepsilon_{556} - \varepsilon_{600} = 34.7\ mM^{-1} \cdot cm^{-1}$ [21] or for cytochrome *b*-559 in particular, a value of $23.4\ mM^{-1} \cdot cm^{-1}$ has been given for $\varepsilon_{556} - \varepsilon_{539}$ by Appleby [20]. At first, we measured the spectrum of the pyridine haemochrome derived from the D1/D2 reaction centre complex of Nanba and Satoh and from the Ghanotakis et al. PS II core complex, which contained 4

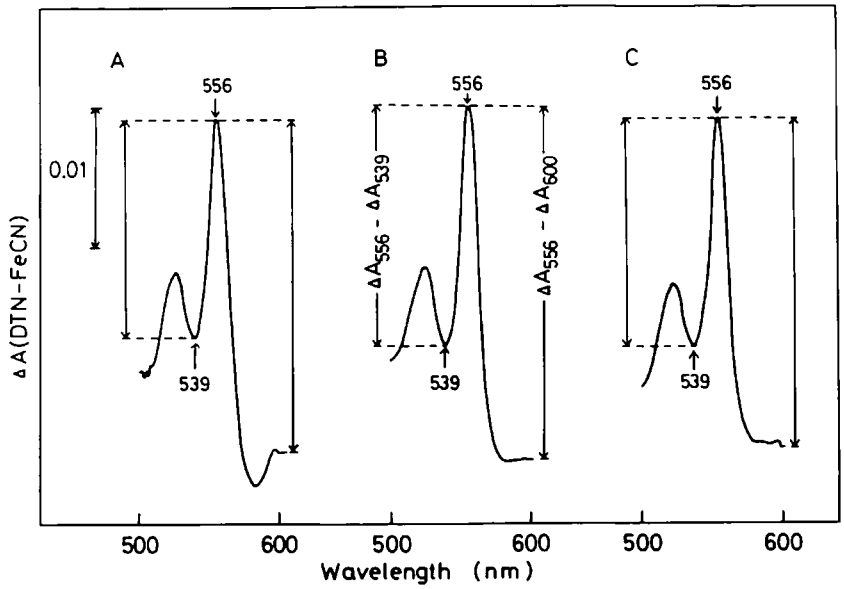

Fig. 1. Dithionite-FeCN spectrum of the pyridine hemochrome derived from (A) PS II core complex prepared according to Ghanaotkis et al. [16] (B) PS II reaction centre prepared according to Nanba and Satoh [5] and (C) purified cytochrome *b*-559. One volume of sample solution was added to 3 volumes of a solution containing 0.1 M NaCl, 0.267 mM $K_3Fe(CN)_6$, 33% vol. pyridine and immediately its absorption spectrum was measured at 20 °C and memorised. Then a few grains of sodium dithionite were added into the same solution and the spectrum measured again. The dithionite-FeCN spectrum was obtained as the difference between them.

Chl *a* and 40 Chl *a* per reaction centre, respectively. For comparison, the dithionite-reduced spectrum of the pyridine haemochrome derived from the purified cytochrome *b*-559 was also measured. As shown in Fig. 1 when the dithionite-reduced minus FeCN-oxidised difference spectra were measured, the traces obtained with both complexes and with the purified cytochrome *b*-559 showed almost identical patterns, which is typical for the reduced pyridine protohaemochrome. Furthermore, the values of $(\Delta A_{556} - \Delta A_{539})/(\Delta A_{556} - \Delta A_{600})$ calcu-

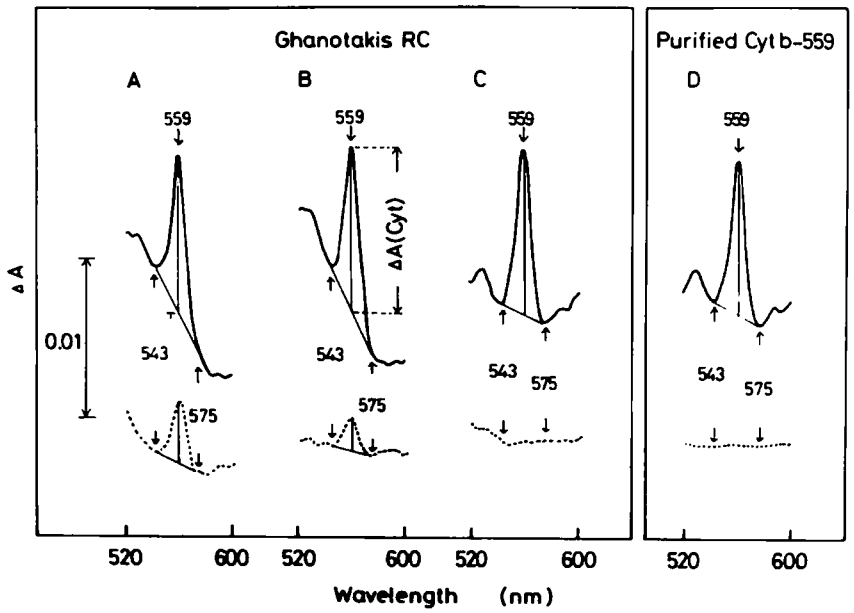

Fig. 2. Effect of Triton X-100 on dithionite-FeCNspectrum (———) and (no addition)-FeCN spectrum (- - -) of a Ghanotakis et al. PS II core complex and purified cytochrome *b*-559. Absorption spectrum of the core complex was measured in solution A (no additions), containing 0.2 mM $K_3Fe(CN)_6$ (A_{FeCN}) and containing 20 mM sodium ascorbate and a few grains of sodium dithionite (DTN) under completely the same condition with or without Triton X-100. Dithionite-FeCN and (no addition)-FeCN spectra were calculated as $A_{DTN} - A_{FeCN}$ and $A_{no\,addition} - A_{FeCN}$, respectively. In the case of purified cytochrome *b*-559, a solution of Tris-HCl (pH 8.0), 0.1% Triton X-100 was used instead of solution A. Spectra of the PS II core complex obtained at 20 °C (A) in the absence of Triton X-100. (B) in the presence of 0.04% Triton X-100. (C) in the presence of 0.1% Triton X-100. (D) dithionite-FeCN and (no addition)-FeCN spectra of purified cytochrome *b*-559 obtained in the presence of 0.1% Triton X-100.

lated from the dithionite-FeCN spectra obtained with the PS II reaction centre complex, the PS II core complex and the purified cytochrome *b*-559, were 0.68 ± 0.01, 0.66 ± 0.02 and 0.71 ± 0.03, respectively, all of which coincide reasonably well with the corresponding value expected for the reduced pyridine protohaemochrome (0.67) calculated from the extinction coefficients given above (from Refs. 20 and 21). These results suggest that in the spectral region used the effect of pigments existing in the assay solution can be eliminated by measuring the dithionite-FeCN difference spectrum. Consequently, the extinction coefficients reported for the reduced pyridine protohaemochrome can be used for the analysis of the dithionite-FeCN spectrum of the pyridine haemochrome to estimate the concentration of cytochrome *b*-559 in the PS II preparations.

With this conclusion we set out to examine the redox difference spectrum of native cytochrome *b*-559 within the PS II complex focusing on the effect of detergent, such as Triton X-100. Besides the dithionite-FeCN spectrum, (no addition)-FeCN spectrum (difference in the absorbance obtained without redox reagent and with FeCN) was mesured. Fig. 2 shows the dithionite-FeCN and (no addition)-FeCN spectra of the PS II core complex as a function of Triton X-100 concentration in the assay solution. The dithionite-FeCN spectrum for the purified cytochrome *b*-559 is also shown for comparison (Fig. 2D). When the measurement was carried out in the absence or at a low concentration of Triton X-100, the dithionite-FeCN spectrum of the complex was different to that of the purified cytochrome *b*-559. However, when the Triton X-100 was added to the solution at a level of 0.1%, the dithionite-FeCN spectrum of the PS II complex was converted into the spectrum of purified cytochrome *b*-559 concomitant with a slight increase in the intensity, while the intensity of the (no addition)-FeCN spectrum of the complex decreased with Triton X-100 concentration and became zero at 0.07%. Similar data was obtained with PS II particles prepared by the method of Kuwabara and Murata. Since the shape of each redox difference spectrum varied with the concentration of Triton X-100 at lower than 0.05%, we estimated the net difference absorbance at 559 nm attributed to cytochrome *b*-559, ΔA_{559} (cytochrome), as $\Delta A_{559} - \frac{1}{2}(\Delta A_{543} + \Delta A_{575})$, where ΔA_{559}, ΔA_{543} and ΔA_{575} represent the apparent difference absorbance at 559, 543 and 575 nm, respectively. In Fig. 3, the values of ΔA_{559} obtained from the dithionite-FeCN and (no addition)-FeCN spectra are plotted against the Triton X-100 concentration using the data obtained with the Ghanotakis et al. PS II core complex. In the case of the dithionite-FeCN, the value of ΔA_{559} (cytochrome) slightly increased with increasing Triton X-100 concentration up to 0.05% and then remained constant between 0.05% and 0.2%. In contrast the (no addition)-FeCN, ΔA_{559} (cytochrome) difference

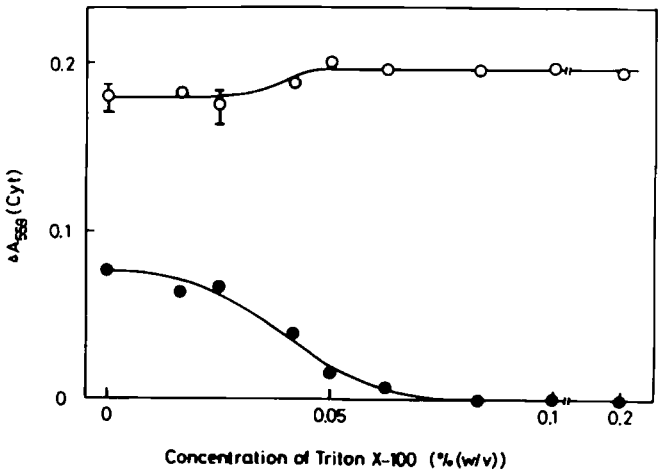

Fig. 3. Effect of the Triton X-100 concentration on ΔA_{559} (cytochrome) determined from the dithionite-FeCN spectrum (open circles) and (no addition)-FeCN spectrum (closed circles) of the PS II core complex of Ghanotakis et al. [16].

decreased with an increase in the Triton X-100 concentration, indicating that the high-potential form of cytochrome *b*-559 in the complex is converted successively into the low-potential form with increasing concentration of Triton X-100. It should be mentioned here that in all preparations of the Ghanotakis et al.-type PS II core complex examined in the present work, about 40% of total cytochrome *b*-559 was in a high-potential form as long as the Triton X-100 concentration was kept lower than 0.02%. This finding is not consistent with the results reported by Ghanotakis et al. [16], where cytochrome *b*-559 in the high-potential form was not detected.

Determination of the redox difference extinction coefficient of cytochrome *b*-559 at 559 nm was carried out by examining the relationship between the in vivo ΔA_{559} (cytochrome) and $\Delta A_{556} - \Delta A_{539}$ of the pyridine haemochrome obtained from the corresponding dithionite-FeCN spectrum. The value of ΔA_{559} (cytochrome) was determined in the presence of 0.1% Triton X-100, because the spectra obtained with PS II preparations in the presence of 0.1% Triton X-100 were almost identical to the spectrum of purified cytochrome *b*-559 and were highly reproducible on the repeated runs. The results obtained are summarised in Fig. 4 as plots of ΔA_{559} (cytochrome) against $DA_{556} - \Delta A_{539}$ for pyridine haemochrome. The plots, including the data for the purified cytochrome *b*-559, followed a straight line passing through the origin. From the slope of the line, the redox difference extinction coefficient of cytochrome *b*-559 at 559 nm, ε_{559} (cytochrome) was determined as 23.4 + 0.3 $mM^{-1} \cdot cm^{-1}$ by using $\varepsilon_{556} - \varepsilon_{539} = 23.4$ $mM^{-1} \cdot cm^{-2}$ reported for reduced protohaemochrome [20]. The value obtained is about 10% larger than the value of 21.0 $mM^{-1} \cdot cm^{-1}$ reported in Ref. 22. The discrepancy between them is due presumably to the

difference in experimental conditions, that Triton X-100 was added at a level of 0.1% in the solution for spectrum measurement in the present work even for the purified cytochrome *b*-559 preparation, but not in the work of Garewal and Wasserman [22]. As mentioned before, the addition of 0.1% Triton X-100 to assay solution results in increasing ΔA_{559} (cytochrome) by 10% (see Fig. 3).

Number of cytochrome b-559 per PS II reaction centre

In order to determine the number of cytochrome *b*-559 involved in one PS II reaction centre the concentrations of cytochrome *b*-559 and of reaction centre in given solutions were measured simultaneously with different preparations of the PS II reaction centre and core complexes and with preparations of PS II particles prepared by the method of Kuwabara and Murata. The concentration of cytochrome *b*-559 was determined by using the value of ε_{559} obtained from the dithionite-FeCN spectrum in the presence of 0.1% Triton X-100 with millimolar extinction coefficient of 23.4 $mM^{-1} \cdot cm^{-1}$ and/or by using the value of $\Delta A_{556} - \Delta A_{539}$ obtained from the dithionite-FeCN spectrum of the pyridine hemochrome with the millimolar extinction coefficient of 23.4 $mM^{-1} \cdot cm^{-1}$. The concentration of reaction centre was determined from the level of pheophytin *a* (Pheo *a*) with the assumption that there are two Pheo *a* per PS II reaction centre.It is very difficult to estimate the concentration of cytochrome *b*-559 and Pheo *a* molecules in the K-M PS II particle preparations accurately because of the high level of Chl *a* and other pigments present, such as carotenoids. On the other hand, although the accuracy of the corresponding data

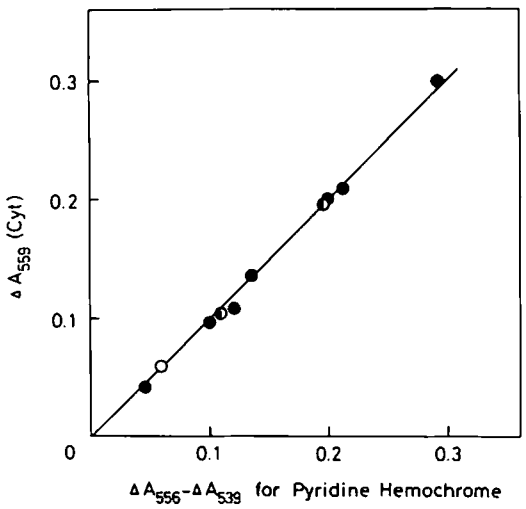

Fig. 4. Relationship between ΔA_{559} (cytochrome) and $\Delta A_{556} - \Delta A_{539}$ of pyridine haemochrome obtained with the data for PS II reaction centre complex (closed circles), PS II core complex (half closed circles) and the purified cytochrome *b*-559 (open circles). ΔA_{559} (cytochrome) was calculated from the dithionite-FeCN spectrum of each sample measured in the presence of 0.1% Triton X-100.

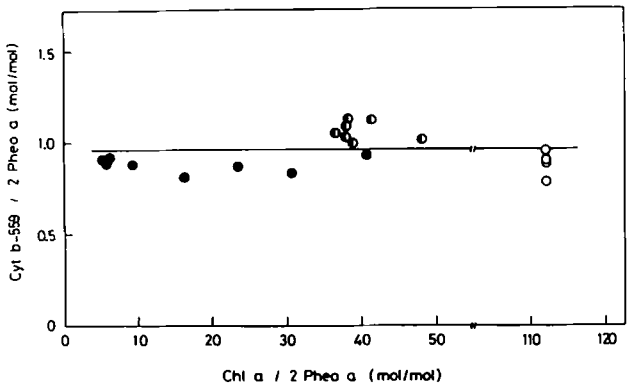

Fig. 5. Number of cytochrome *b*-559 per reaction centre (i.e., per 2 Pheo *a*) as a function of number of Chl *a* per reaction centre in differently prepared PS II reaction centre complexes and PS II particles. (closed circles) PS II reaction centre according to Nanba and Satoh [5]; (half closed circles) PS II core complex according to Ghanotakis et al. [16]; (open circles) PS II particles according to Kuwabara and Murata [15].

obtained with the reaction centre and core complexes is considerably better (because the level of background pigments has been significantly reduced) it is possible that some cytochrome *b*-559 is removed from these purified complexes during the detergent treatments. In order to avoid this uncertainty we examined the stoichiometry of cytochrome *b*-559 in the various types of preparation at different purification stages. In the case of the Nanba and Satoh type reaction centre complex, the level of purification was controlled by varying the time for washing the DEAE column with 0.2% Triton X-100 solution between 1 and 24 h. By this method we obtained preparations containing 5.3 through to 40.7 molecules of Chl *a* per reaction centre (i.e., based on two Pheo *a* molecules per reaction centre). The number of cytochrome *b*-559 per reaction centre obtained with these preparations is shown in Fig. 5 as a function of those of Chl *a*. Apparently column chromatographic treatment of the PS II particles by using DEAE Toyopearl and solution E, which contains 0.2% Triton X-100, to prepare the Nanba-Satoh reaction centre complex, did not lead to the removal of cytochrome *b*-559 from the resulting reaction centre complex. As can be seen clearly in Fig. 5 the stoichiometry of cytochrome *b*-559 in the reaction centre complex was kept at the same level as in the K-M PS II particles which was the starting material. Fig. 5 also shows the treatment of the PS II particles with octylglucoside in preparing the PS II core preparation of Ghanotakis et al. [16] and seems to result in a slight increase in the number of cytochrome *b*-559 per reaction centre. This increase, however, is less than 15%. The number of cytochrome *b*-559 per reaction centre determined as an average of the data shown in Fig. 5 to be 0.97 ± 0.10. From these results we conclude that the stoichiometry of cytochrome *b*-559 per PS II reaction centre is 1 rather than 2.

Discussion

We have set out to try to solve the controversy regarding the stoichiometry of cytochrome *b*-559 per PS II reaction centre. We have based our stoichiometry on the assumption that each PS II reaction centre contains two pheophytin molecules. Such an assumption is not unreasonable bearing in mind the analogy between the structure of the purple bacterial and the PS II reaction centres [23,24]. Our conclusion that there is one cytochrome *b*-559 per reaction centre is consistent with the observations of a number of workers, including Sandusky et al. [13], Franzen et al. [14], DeVitry et al. [25]. Decker et al. [26] and Rémy and Ambard-Brettville [27]. It also agrees with the previous measurements of Gounaris and Barber [28] and Gounaris et al. [29] using a PS II core preparation isolated with Triton X-100.In these papers it was reported that there is about one cytochrome *b*-559 per two pheophytin molecules [28] and that there was a 1 : 1 : 1 stoichiometry between the α-subunit of cytochrome *b*-559 and the D1, D2 polypeptides [29] per PS II reaction centre. These conclusions contrast with the claims of others that there are two cytochrome *b*-559 per PS II reaction centre [11,12,30,31]. The origin of this discrepancy is unclear. Many of these workers have used $\varepsilon_{559-570} = 15\ mM^{-1} \cdot cm^{-1}$ based on that given in the review by Cramer and Whitmarsh [1]. This lower extinction coefficient would tend to give a slightly higher cytochrome level but would not double the concentration. It is difficult therefore to postulate an obvious reason for the controversy except that our arguments hinge on the basis of two pheophytins per PS II reaction centre and that those analysed in our samples are the functional species. In this respect it is relevant to consider earlier data obtained by Gounaris and Barber with the Triton X-100 derived PS II core preparations mentioned above. Not only did they measure one cytochrome *b*-559 per two pheophytins in their preparation but also one cytochrome *b*-559 per two plastoquinones. Based on the bacterial reaction centre structure and the recent findings that the electron donors D and Z are not plastoquinone molecules [32,33] it would be predicted that in each PS II reaction centre there are two plastoquinones per two pheophytins. Therefore our conclusion that there is only one cytochrome *b*-559 per PS II reaction centre is reinforced by our earlier work as well as being consistent with previous analyses by us on the isolated D1/D2/cytochrome *b*-559 complex [10].

Acknowledgements

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