Comparison of the D1/D2/cytochrome b559 reaction centre complex of photosystem two isolated by two different methods

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Photosystem 2 reaction centre complexes prepared either by solubilisation with Triton X-100 and subsequent exchange into dodecyl maltoside or by a procedure involving a combination of dodecyl maltoside and $LiClO_4$, were characterised in terms of chlorophyll *a*, pheophytin *a*, β-carotene and cytochrome b559 content. Time-resolved chlorophyll fluorescence decay kinetics were measured using both types of complexes. Our data show that the isolated photosystem two reaction centre complex contain, for two pheophytin *a* molecules, close to six chlorophyll *a*, two β-carotene and one cytochrome b559. No major differences were observed in the composition or the kinetic characteristics measured in the samples prepared by the different procedures. Time-resolved fluorescence measurements indicate that more than 94% of the chlorophyll *a* in both preparations is coupled to the reaction centre complex.

Photosystem 2; Reaction centre; Pigment; Cytochrome b559; Time-resolved chlorophyll fluorescence measurement

1. INTRODUCTION

The isolation of a photochemically active reaction centre of photosystem two (PS2) [1,2] confirmed the suggestion that the PS2 polypeptides, D1 and D2, function in a similar way to the L and M subunits of the reaction centre of purple bacteria [3,4]. X-Ray crystallography confirmed unequivocally that the L and M subunits bind four bacteriochlorophyll *a*, two bacteriopheophytin and one carotenoid [5,6]. Based on amino acid sequence comparisons between the D1 and D2 polypeptides and the L and M subunits, histidine residues have been identified in the D1 and D2 polypeptides which are likely to be involved in binding both the chlorophyll *a* (Chl *a*) of the primary electron donor P680, and the non-haem iron [3,7]. The situation regarding ligands for the accessory Chl *a* is less clear, since there are several histidine residues which can be identified as potential candidates but none of these seem to correspond to the histidine residues which bind the accessory bacteriochlorophyll to the L and M subunits [5,8].

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Abbreviations: PS2, photosystem 2; RC-1, reaction centre prepared as in [14,15]; RC-2, reaction centre prepared as in [9]; Cyt b559, cytochrome b559; Chl *a*, chlorophyll *a*; Pheo *a*, pheophytin *a*; CP47, 47 kDa chlorophyll binding protein encoded by the *psb*B gene; D1 polypeptide, product of the *psb*A gene; D2 polypeptide, product of the *psb*D gene

Although initial analyses indicated that the isolated D1/D2 complex bound 4–5 chlorophylls [1,2] there have been more recent reports that this level can be higher [8–10]. Moreover, it has also been suggested that the level of β-carotene is higher in the isolated complex than originally thought [9]. Related to these findings is the controversy regarding the cytochrome b559 (Cyt b559) content of the isolated D1/D2 complex [11,12]. Indeed in a recent paper, Dekker et al. [9] assumed that there were two molecules of this cytochrome per complex and therefore suggested that the isolated reaction centre bound 10–12 Chl *a*. Such a Chl *a* to Cyt b559 ratio is similar to that found previously [1,11,13] but, unlike other groups, Dekker et al. estimated about two pheophytin *a* (Pheo *a*) per two Cyt b559. Only in the case of the D1/D2 complex isolated from the cyanobacterium, *Synechocystis* 6803, did we report a high chlorophyll-to-pheophytin ratio, namely 10–12 Chl *a* per 2 Pheo *a* [10]. Based on this stoichiometry, however, we estimated one Cyt b559.

In order to clarify the situation we have isolated the PS2 reaction centre using the procedure of Dekker et al. [9] and compared its chemical composition and photochemical activities with that of the reaction centre isolated using a modified version [14] of that first reported by Nanba and Satoh [1].

Photochemical activities were also determined from time-resolved fluorescence data. Time-resolved fluorescence measurements of isolated reaction centre complexes, prepared as in [14,15], have shown a lifetime of 37 ns, contributing 44% of the total fluorescence. This lifetime has been assigned to the

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singlet excited state of P680 (P680*) in equilibrium with the primary radical pair, $P680^+Pheo^-$ [16,17]. The remainder of the fluorescence is predominantly a 6.5 ns component attributed to chlorophyll which is energetically uncoupled from the process of charge separation.

2. MATERIALS AND METHODS

All photosystem 2 complexes were isolated from pea (*Pisum sativum*) thylakoid membranes. The D1/D2/cyt b559 reaction centre complex (RC-1) was derived by Triton X-100 solubilisation of PS2-enriched membranes as described by Chapman et al. [14] with the second chromatographic separation carried out in buffer containing 2 mM dodecyl maltoside [15]. A PS2 complex capable of oxygen evolution was prepared as described by Ghanotakis et al. [18] and this PS2 preparation was used to produce a CP47/D1/D2/cyt b559 complex and a D1/D2/cyt b559 reaction centre complex (RC-2) as described by Dekker et al. [9]. Room temperature absorption and fluorescence emission spectra were recorded as described previously [2]. Total pigment levels were determined as in [9] using the extinction coefficient of 74000 $M^{-1} \cdot cm^{-1}$ for absorbance at 675 nm. The absorption spectra for total pigments in acetone were taken after extraction as in [8].

Quantitative analyses of the individual pigments were carried out by high-performance liquid chromatography. Pigments were extracted with chloroform/methanol (2:1, v/v) according to [19], samples dried under N_2, redissolved in methanol, and 100 μl samples of the extract containing about 2 nmol chlorophyll assayed by reverse-phase chromatography using a Spherisorb RP-18 column with an isocratic solvent system of acetonitrile/methanol (3:2, v/v) and a flow rate of 1.0 $ml \cdot min^{-1}$. Chl *a* and Pheo *a* were monitored continuously at 663 nm and β-carotene at 455 nm to give integrated peak areas which were compared with those from standards prepared using known weights of chlorophyll *a* and β-carotene. Pheo *a* standards were prepared by pheophytinisation of known amounts of Chl *a* (1–20 $nmol \cdot ml^{-1}$) by the addition of 150 μl methanolic solution of Chl *a* to 2 μl 0.5 M HCl. The concentrations of standards were confirmed using the extinction coefficients published in [20] with absorbance measurements in 100% methanol, 100% acetone and 80% acetone in water. The cytochrome b559 content was measured from the reduced (dithionite) minus oxidised (ferricyanide) difference absorption spectra using an extinction coefficient of 17500 $M^{-1} \cdot cm^{-1}$ as determined in [21] and used by Dekker et al. [9]. However, it should be noted that both higher and lower extinction coefficients have been given by others, as discussed in [11].

Excited singlet-state lifetimes were measured using time-correlated single photon counting. The apparatus consisted of a cavity dumped Rhodamine 6G dye laser synchronously pumped by a mode-locked Coherent Antares Nd-YAG. This provided a 3.7 MHz train of 8 ps pulses at 615 nm with an average power of 20 mW. Emission at 682 nm was selected with a Hilger-Watts monochromator and detected with a Hamamatsu R1564.U01 microchannel plate photomultiplier tube. The instrument response function was measured to be 120 ps at full width half maximum (FWHM). Lifetimes were calculated by iterative re-convolution based on a Marquardt fitting algorithm. All decays were measured to 20000 counts in the peak channel and the quality of fits was judged using a reduced χ^2 criterion and plots of the weighted residuals. Further details of the analysis technique can be found in [22] and the assignment of lifetimes in [16,17]. All samples were resuspended to give a concentration of 10 μg/ml chlorophyll and were maintained at 4°C at all times. Anaerobic conditions were achieved in all samples by the addition of 5 mM glucose, 0.1 mg/ml glucose oxidase and 0.05 mg/ml catalase. Samples were stirred during time-resolved measurements.

3. RESULTS

Using the same type of oxygen-evolving PS2 core particles as those reported in [18] and following the procedures described in [9], we have prepared a PS2 preparation containing CP47, D1, D2 polypeptides and

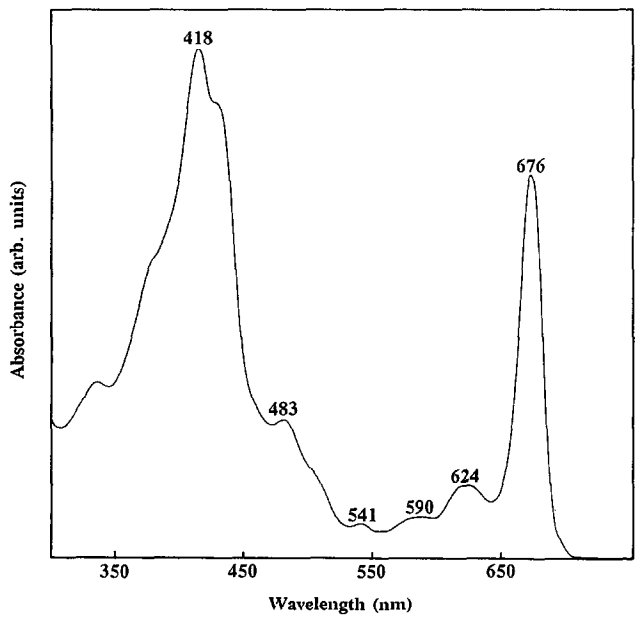

Fig. 1. Absorption spectra of PS2 reaction centre RC-2, prepared as in [9].

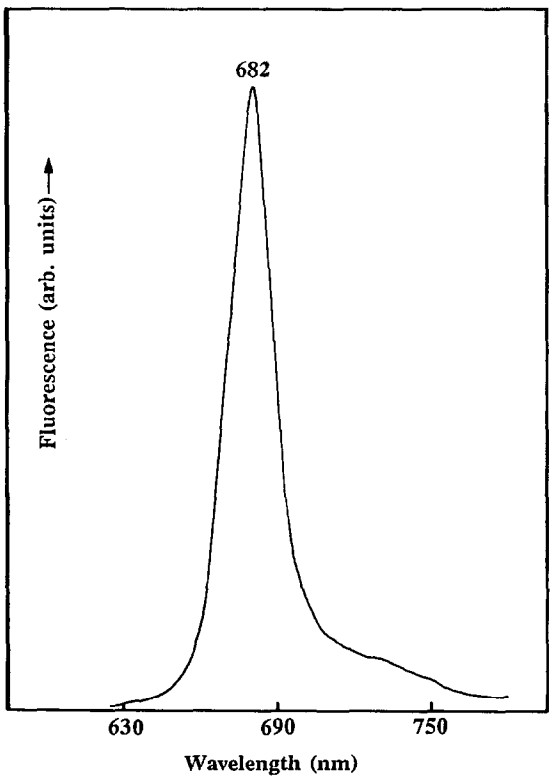

Fig. 2. Room temperature fluorescence emission spectrum of PS2 reaction centre RC-2 prepared as in [9].

cytochrome b559 and used this to derive a D1/D2/cyt b559 complex by removal of the 47 kDa protein (RC-2). The polypeptide composition of the different preparations was very similar to that reported in [9]. Assuming that there was a 100% recovery of Pheo *a* during the isolation of RC-2 from the CP47/D1/D2/cyt b559 complex we estimate a yield of 41% for the total pigment and 87% for the cyt b559 content. This is in very good agreement with the values reported in [9].

The absorption and room temperature fluorescence emission spectra of the RC-2 preparation are shown in Figs 1 and 2, respectively. The absorption spectrum is very similar to those previously published for preparations made by the same (RC-2) technique [9] and by the type of method used in our laboratory [23]. The room temperature fluorescence emission spectrum of the RC-2 preparation has not been previously described but is essentially the same as that reported for RC-1 [2,16] with a maximum emission at 682 nm. The close similarity of the RC-1 and RC-2 preparations was also emphasised by the absorption spectra of the pigments extracted and resuspended in acetone. A typical spectrum is shown in Fig. 3 and is much like that given in [8].

To further compare and characterise the isolated RC-1 and RC-2 preparations we carried out chemical determinations of all pigments and measured Cyt b559 from its reduced-oxidised difference spectrum. Our data are shown in Table I and expressed on the basis of two Pheo *a* molecules, giving close to six Chl *a*, two *β*-carotene and one Cyt b559 in RC-2 complexes prepared as in [9]. Analyses of RC-1 preparations gave about the same Chl *a* level and only slightly higher cytochrome levels. In the CP47/D1/D2/Cyt b559 preparation we estimate, on the basis of two Pheo *a* molecules, about 18 Chl *a*, two *β*-carotene and one Cyt b559.

Table I

Pigment and cytochrome b559 composition of isolated PS2 particles (values shown ± SD)

	Chl *a*	Pheo *a*	*β*-Carotene	Cyt b559
RC-1 (as in [14])	5.81 ± 0.64	2.00	2.18 ± 0.23	1.42 ± 0.27
RC-2 (as in [9])	6.21 ± 0.13	2.00	1.96 ± 0.04	1.04 ± 0.05
CP47/D1/D2/ cyt b559	18.00	2.00	1.80	1.19

We have carried out time-resolved chlorophyll fluorescence emission spectroscopy to further compare the two types of isolated reaction centre and to determine if all the chlorophyll molecules associated with the complexes have roles in reaction centre function. Previous measurements of this type on RC-1 preparations [16,17] were made in a buffer of 50 mM Tris-HCl, pH 8.0, 2 mM dodecyl maltoside (buffer A). However, the RC-2 preparation of Dekker et al. [9] was resuspended in a buffer of 20 mM Bis-Tris, pH 6.5, 20 mM NaCl, 10 mM $MgCl_2$, 1.5% taurine, 0.03% dodecyl maltoside (buffer B). To ensure that we made a rigorous comparison of the two preparations, we first measured RC-1 in both buffers and then compared RC-1 with RC-2 using buffer B.

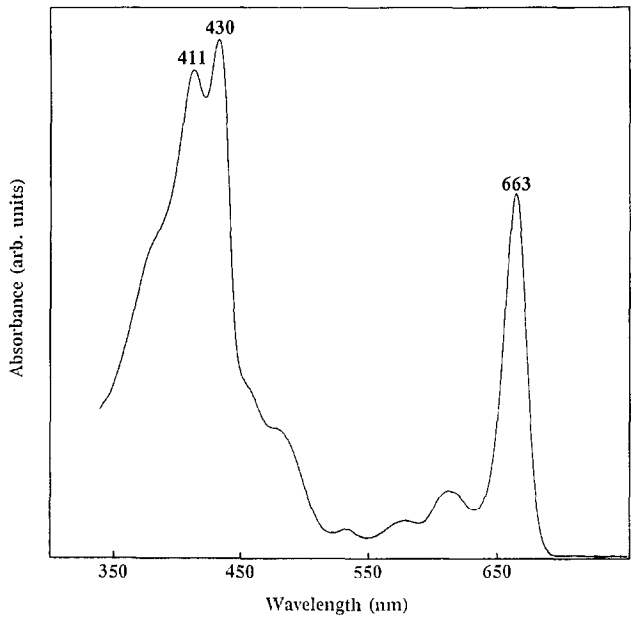

Fig. 3. Room temperature absorption spectrum of an acetone extract of the PS2 reaction centre RC-1 prepared as in [14] recorded in 100% acetone.

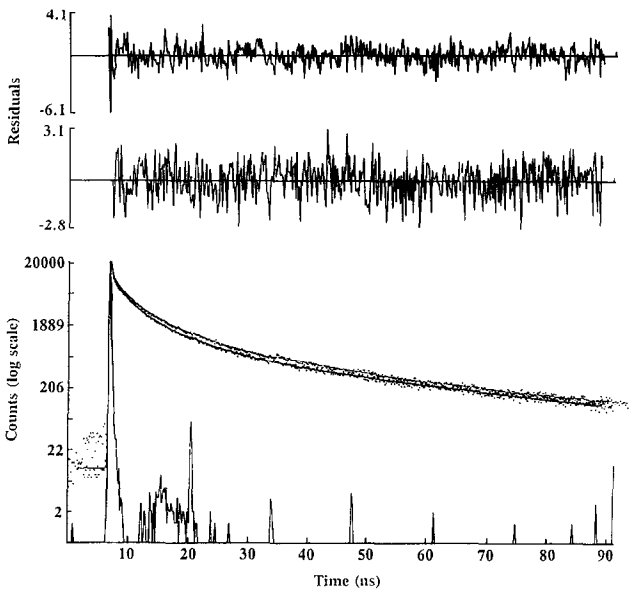

Fig. 4. Time-resolved chlorophyll fluorescence decay kinetics of isolated reaction centres RC-1 (upper trace) in buffer A and RC-2 (lower trace) in buffer B. Also shown are the residuals (upper RC-2; lower, RC-1) for a 4-exponential fit to the data.

Table II

Fluorescence decay characteristics and derived parameters of isolated PS2 reaction centre complexes

	t_1 (ns)	F_1 (%)	F_2 (%)	ΔG (eV)	Chl uncoupled from electron transfer (%)
RC-1 (as in [14] resusp. in buffer A)	36.5 ± 2.5	44 ± 4	40 ± 5	−0.110 ± 0.003	5.6 ± 0.6
RC-1 (as in [14] resusp. in buffer B)	35.7 ± 4.3	52 ± 6	33 ± 9	−0.102 ± 0.004	5.1 ± 0.6
RC-2 (as in [9] resusp. in buffer B)	31.5 ± 2.0	42 ± 4	36 ± 4	−0.110 ± 0.003	4.4 ± 0.8

t_1 is the lifetime of the charge recombination fluorescence, F_1 is the percentage yield of fluorescence associated with charge recombination, F_2 is the percentage yield of fluorescence associated with chlorophyll which is uncoupled from the charge separation process (see [16,17]), ΔG is the free energy gap between the radical pair and the singlet excited state of P680 calculated as in [17]. All decays were analysed to 4 exponentials with the uncoupled chlorophyll lifetime fixed to 6.5 ns; the two shorter fluorescence components are not shown here (see [17])

A comparison of typical time-resolved fluorescence decays of RC-1 and RC-2 is given in Fig. 4. Table II shows fluorescence decay characteristics and derived parameters of RC-1 in buffers A and B and also of RC-2 resuspended in buffer B. The free energy gap between P680* and $P680^+Pheo^-$ was calculated as previously described [17]. The measurements indicate that the fluorescence decays are similar both qualitatively and quantitatively. Although there seems to be a small difference in the lifetime of the long component, and hence in the lifetime of the radical pair, between RC-1 and the RC-2, this variation has little effect on the value of the free energy gap. While the absolute value of the free energy gap may have some systematic errors associated with it [17], the comparison of the free energy gaps obtained by this method can give a useful indication of variations in the state of a preparation and the results obtained here suggest that differences between the free energy gap in the RC-1 and RC-2 complexes are less than 10%.

The overall degree of reaction centre integrity is probably more usefully represented by the percentage yield of the fluorescence due to charge recombination (F_1 in Table II), compared with that from chlorophyll which is uncoupled from the electron transfer (F_2 in Table II). It has previously been demonstrated [16,17] that values of F_2, typical of those shown in Table II, represent emission from less than 6% of the total chlorophyll in the preparation. This indicates that over 94% of the chlorophyll is functionally coupled to the reaction centre.

From Table II it can be seen that there were only slight differences due to the two methods of preparation or due to the different buffers. However, when taking fluorescence decay measurements with RC-1 and RC-2 resuspended in buffer B it was clear that they both showed markedly improved stability to light compared with preparations suspended in buffer A (data not shown). The reaction centres in buffer B showed a decrease in the fluorescence yield of radical pair recombination of about 20% after exposure to 20 mW irradiation at 615 nm for 20 min (under anaerobic conditions), while in buffer A the result of the same preillumination conditions was a decrease of about 70%.

4. DISCUSSION

We have compared the composition and fluorescence properties of the PS2 reaction centre isolated from higher plants either by a method essentially the same as that first reported by Nanba and Satoh [1] or by the new method developed by Dekker et al. [9]. Within experimental error we do not detect any major differences in the composition or photochemical properties of the two types of preparation. Our results strongly support the recently published data of Kobayashi et al. [8] that based on the assumption that there are two Pheo *a* within the PS2 reaction centre, the isolated complex binds 6 chlorophyll *a* and two *β*-carotene molecules. We also confirm our previous conclusion [11] that based on the same assumption regarding the level of pheophytin *a*, the isolated reaction centre complex seems to bind only one, rather than two, cytochrome b559.

At first sight our conclusions appear to contrast with those of Dekker et al. [9]. However, these workers based their claim that the RC-2 preparation binds 10–12 Chl *a* on the assumption that there are two Cyt b559 per reaction centre. Clearly if this assumption is incorrect and there is one Cyt b559 per reaction centre, then the Chl *a* level they find is consistent with the conclusion of ourselves and Kobayashi et al. [8] that there are 6 Chl *a* per reaction centre. In fact, a discrepancy does occur with the level of Pheo *a* which they reported. Their claim, however, that there are only 2–3 molecules of Pheo *a* per 10–12 Chl *a* is not based on a rigorous chemical analysis of the pheophytin content but on an interpretation of the absorption spectrum of the isolated RC-2.

Putting aside the above controversy it should be noted that all recent compositional analyses have been

carried out on PS2 reaction centres isolated from higher plants by modifications of the earlier methods and using complexes which are more stable than those originally analysed [1,2,13]. It now seems that with these new preparations the isolated PS2 reaction centre complex of higher plants differs from the purple bacterial reaction centre in that it binds two carotenoid molecules and has two additional accessory chlorophylls. The significance of these differences has yet to be studied further. Our time-resolved fluorescence lifetime measurements do indicate, however, that these additional chlorophylls are unlikely to be contamination by unbound pigments since they do not have lifetimes indicative of uncoupled chlorophyll. The presence of two carotenoid molecules is also puzzling since flash absorption studies of RC-1 and RC-2 preparations ([24] and unpublished observation) indicate that both types of preparation form a P680 triplet state but that in neither case is this triplet quenched by the bound carotenoids.

An interesting observation which emerged from these studies is that isolated PS2 reaction centres were found to be more resistant to photodamage in buffer B as compared with buffer A. The reason for this difference is currently under investigation.

No major differences were observed in the kinetic characteristics measured in RC-1 and RC-2 preparations. Time-resolved fluorescence measurements indicate that more than 94% of the chlorophyll *a* present in both these preparations is associated with primary charge separation.

Our analyses suggest that the dissimilarities in the pigment composition of the isolated reaction centres of PS2 from higher plants and purple bacteria are not as great as suggested by Dekker et al. [9] but are in agreement with the recent findings of Kobayashi et al. [8]. The situation regarding the composition of the D1/D2/cyt b559 complex isolated from the cyanobacterium, *Synechocystis* 6803, has yet to be reconciled with the above conclusions but worthy of note is the fact that this complex was found to bind two carotenoids per two pheophytin *a* molecules [10] in agreement with the new findings presented here and in [8]. Our results still suggest that there is probably one Cyt b559 per reaction centre although the results of an earlier paper [25] and also the recent paper of Shuvalov et al. [12], suggest that there is a redox heterogeneity in the population of this cytochrome.

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