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Characterization of a D1–D2–cyt *b*-559 complex containing 4 chlorophyll *a*/2 pheophytin *a* isolated with the use of $MgSO_4$

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Abstract

A D1–D2–cyt *b*-559 complex containing 4 chlorophyll *a*, 1 β-carotene and 1 cytochrome *b*-559 per 2 pheophytin *a* has been isolated from spinach with 30% yield using a Q-Sepharose Fast-Flow anion-exchange column equilibrated with 0.1% Triton X-100, 10 mM $MgSO_4$ and 50 mM Tris-HCl (pH 7.2). The preparation was then stabilized with 0.1% dodecyl-β-D-maltoside. This method gave a yield 10 times higher than that using a Fractogel TSK-DEAE 650(S) column equilibrated with 0.1% Triton X-100, 30 mM NaCl and 50 mM Tris-HCl (pH 7.2). The PS II RC complex was characterized using absorption and fluorescence spectroscopy at 277 and 77 K. A selective reversible bleaching under reducing conditions with maximum at 682 nm, associated with pheophytin *a* reduction, and light-induced absorption differences with a lifetime of 1.0 ms, ascribed to the triplet state of P680 were measured and indicated that the isolated D1–D2–cyt *b*-559 complex is active in charge separation. The results are compared with the data obtained for a PS II RC preparation containing 6 chlorophyll *a*, 2 β-carotene and 1 cyt *b*-559 per 2 pheophytin *a*.

Key words: Photosystem II; Reaction center; Pigment composition

1. Introduction

Since the first report on the isolation and characterization of the photosystem (PS) II D1–D2–cyt *b*-559 reaction center (RC) complex from spinach [1], research on fast and simple procedures which yield stable samples on a preparative scale has been intensified. The isolation of this pigment–protein complex has also been described for *Pisum sativum* [2], *Spirodella oligorrhiza* [3], *Beta vulgaris* [4] and *Laminaria saccharina* [5]. Basically, the method developed by Nanba and Satoh [1] yields, after a long-term Triton X-100 exposure, a PS II RC stoichiometry of 4–5 chlorophyll *a*, 1 β-carotene and 1 cytochrome *b*-559 per 2 pheophytin *a*. Several reports have shown that the D1–D2–cyt *b*-559 complex is fairly unstable in the presence of Triton X-100 [3,6] and some alternative methods have been described to obtain more stable preparations [7–10] which contain 6 chlorophyll *a*, 2 β-carotene, 1 cytochrome *b*-559 per 2 pheophytin *a*. At present, many research groups work with such preparations assuming that the 6 Chl *a*/2 Pheo *a* stoichiometry represents the intact D1–D2–cyt *b*-559 complex [11–13]. However, it has been shown that an extensive Triton X-100 exposure leads to the loss of pigment molecules, i.e. two chlorophyll and one β-carotene molecules [4,14]. A reduced number of pigments in the PS II RC could be advantageous for the characterization and assignment of the spectroscopic properties of the chromophores, which is essential for understanding the functional details of the PS II RC. One of the major questions in this respect pertains to the minimal number of pigments required for charge separation activity. Presumably, two Chl *a* molecules, analogous to the special pair of BChl *a* molecules in purple bacterial RC, constitute the primary electron donor P680 and one Pheo *a* molecule acts as the primary electron acceptor.

Optical spectroscopic studies on the PS II RC are complicated due to strongly overlapping absorption spectra of all pigments. Some authors have concluded that excitonic interactions exist among all the pigments within the PS II RC. Several recent reports have proposed assignments to Q_Y transitions of the chlorins in the PS II RC [15,16], but, actually, the assignment of bands to a specific group of chromophores is subject to debate.

Knowledge of the primary photochemistry and structure of the PS II RC is increasing with the use of time-resolved spectroscopies. Recently, the study of the primary kinetics in 6 Chl-PS II RC has been intensified [17–19], however, only few data have been obtained with RCs containing about 4 chlorophylls. In order to apply

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Abbreviations: Chl, chlorophyll; cyt, cytochrome, CP47, chlorophyll–protein in the core complex of photosystem II with apparent molecular mass of 47 kDa; D1–D2–cyt *b*-559: reaction center complex of photosystem II; FWHM, full width at half maximum; PAGE, polyacrylamide gel electrophoresis; Pheo, pheophytin; PS II, photosystem II; P680, primary electron donor; RC, reaction center; SDS, sodium dodecyl sulfate; Tris, Tris(hydroxymethyl)aminomethane.

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these techniques relatively high amounts of RC particles are required (i.e. 0.6–1.0 mg Chl), but the methods which are available so far to prepare RCs with a reduced pigment content give only a very low yield [14,16]. For this reason it is important to develop new methods to prepare a PS II RC complex with 4 Chl *a*/2 Pheo *a* with higher yields. We report here a new procedure to isolate the D1–D2–cyt *b*-559 complex containing 4 Chl *a* per 2 Pheo *a* with 30% yield, demonstrate its functional integrity and compare it with the corresponding data for the 6 Chl *a*/2 Pheo *a* preparation. The pigment composition, absorbance and fluorescence spectra and measurements of charge separation and triplet formation are presented.

2. Materials and methods

2.1. Preparation of the D1–D2–cyt b*-559 complex*

PS II membranes were isolated from spinach leaves according to the method of Berthold et al. [20] with minor modifications as described by van Leeuwen [13]. For the isolation of the D1–D2–cyt *b*-559 complex containing 4 Chl *a*/2 Pheo *a*, PS II membranes were diluted in 50 mM Tris-HCl (pH 7.2) to a concentration of 1 mg Chl/ml and solubilized in 4% Triton X-100 (w/v) for 1 h in the dark at 4°C. The non-solubilized material was removed by a centrifugation step at 100,000 × *g* for 1 h to avoid clogging of the column. The resultant supernatant was loaded onto a 3.0 × 21 cm Q-Sepharose Fast-Flow column (Pharmacia LKB) equilibrated with 50 mM Tris-HCl (pH 7.2), 10 mM $MgSO_4$ and 0.1% Triton X-100. The column was washed with the same buffer until the absorbance of the eluate was 0.05 cm^{-1} at 670 nm and absorbances $A_{417} > A_{435}$ (indicating RC particles are eluting). At this point detergent was exchanged from 0.1% Triton X-100 to 0.1% dodecyl-β-D-maltoside in the same buffer until $A_{280} < 0.1$ (indicating Triton X-100 is removed) and the D1–D2–cyt *b*-559 complex was eluted with a gradient of 30–200 mM $MgSO_4$. The main fraction was collected from the column at a concentration of 150 mM $MgSO_4$. All steps were carried out at 4°C in the dark and the sample was stored at 77 K until use. PS II RCs containing 6 Chl *a*/2 Pheo *a* were prepared as in [13]. The yield of the preparation was determined by comparison of the Chl *a* concentration in PS II membranes and in the isolated PS II RC complex. We considered the Chl *a*/RC ratio of 250:1 in PS II membranes [23] vs. 4:1 in our preparation.

2.2. Quantitation of Chl a*, Pheo* a*, cyt* b*-559 and β-carotene*

Chl *a* and Pheo *a* amounts were determined from their absorption spectra in 100% acetone extract with a Perkin-Elmer 340 spectrophotometer as described in [4]. The amount of cyt *b*-559 was calculated from the dithionite-reduced minus ferricyanide-oxidized difference absorption spectra using an extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$ at 559 nm [21]. β-Carotene was determined by HPLC with a Shimadzu chromatograph using a Nucleosil-5-C18 reversed-phase column at room temperature. Pigments were extracted with MeOH/THF (90:10) and sonicated for 2–3 min. The sample was eluted isocratically with MeOH at a flow rate of 0.8 ml/min using a LC 6-A pump. Peaks were monitored by a SPD 6 AU detector at 450 and 415 nm.

2.3. Spectroscopy

Samples were diluted with a buffer containing 50 mM Tris-HCl (pH 7.2) and 0.1% dodecyl-β-D-maltoside. For low temperature measurements glycerol was added to a final concentration of 60% (v/v) before freezing. Absorption spectra were recorded with a Perkin-Elmer 320 spectrophotometer using a 3-mm pathlength cuvette. All measurements at room temperature were carried out under anaerobic conditions, attained by addition of 0.23 mg/ml glucose oxidase, 80 μg/ml catalase and 10 mM glucose to the sample. For measurements at 77 K an Oxford Instruments DN 704 cryostat was used. Corrected fluorescence emission and excitation spectra were carried out with a SPEX Fluorolog spectrometer. Absorption difference spectra upon continuous illumination were measured under the same conditions as in [6]. The D1–D2–cyt *b*-559 complex ($OD_{675.5} = 0.7$) was treated with 1 μM methyl viologen and 10 mM sodium dithionite. Samples were illuminated with white light, passed through a 2 cm pathlength cuvette with water that absorbed unwanted IR radiation The sample temperature was maintained at 4°C.

Flash-induced absorption difference measurements were carried out at 4°C as well, under anaerobic conditions. The excitation source was a Xe-flashlamp that delivered pulses of 20 μs FWHM, with a long tailing of the discharge that affected the measurement for about 250 μs after triggering the flashlamp. The excitation light was filtered with a Schott GG 400 filter of 2 mm thickness. Each measurement presented is the average of 32 flash events. Absorption differences were detected with a Si-photodiode and amplified by a home-built current-to-voltage converter.

2.4. Electrophoresis

SDS-PAGE was carried out as in [22] in a 12.5% and 15–20% acrylamide gel containing 6 M urea. Samples were denatured at room temperature for 1 h in a solution containing 0.05 M Tris-HCl (pH 7.2), 2% (w/v) SDS, 2 M urea, 40 mM dithiothreitol and 20% (w/v) sucrose. Gels were stained with Coomassie brilliant blue.

3. Results

A D1–D2–cyt *b*-559 complex preparation was obtained from PS II membranes of spinach with 30% yield. This yield was calculated assuming the presence of 250 Chl *a*/RC in the PS II membranes [23].

The use of a strong anion-exchange column (Q-Sepharose Fast-Flow) and divalent cations (i.e. $MgSO_4$) for the separation gave a yield 10 times higher than when using a weak anion-exchange column and monovalent cations (i.e. Fractogel TSK-DEAE 650(S) and NaCl) [1,4]. Table 1 summarizes the pigment and cyt *b*-559 content of our RC preparation which correspond well with those previously reported for long-term Triton X-100 treated RC

Table 1
Pigment stoichiometry and cyt *b*-559 content of the 4 Chl *a*/2 Pheo *a* D1–D2–cyt *b*-559 complex purified using a Q-Sepharose Fast-Flow column and $MgSO_4$

	This preparation	RC2 [4]	PSIIRC [1]	PSIIRC [13]
Chl *a*	4.1 ± 0.3	4.1 ± 0.1	5.1 ± 0.5	5.9[a]–6.2 ± 0.3[b]
Pheo *a*	2.0	2.0	2.0	2.0
β-Carotene	0.9 ± 0.1	0.84	1.0 ± 0.3	1.0[c]
cyt *b*-559	1.1 ± 0.2	1.2 ± 0.1	1.3 ± 0.4	1.3[a]–1.2 ± 0.2[b]

[a] Values reported in the original work [13].
[b] Values calculated using the procedure described in section 2.
[c] Value reported in [27].

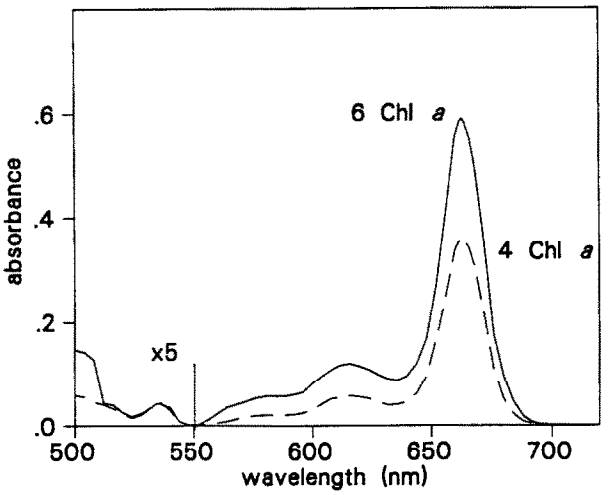

Fig. 1. Absorption spectra of acetone extracts of D1–D2–cyt *b*-559 complexes containing 6 Chl *a* (solid line) and 4 Chl *a* (dashed) per 2 Pheo *a*, normalized at 535 nm, assuming equal Pheo *a* content. Samples were prepared as described in section 2.

complexes [1,3,4]. Our data are also compared with the stoichiometry calculated for a D1–D2–cyt *b*-559 complex containing 6 Chl *a*/2 Pheo *a*. Fig. 1 provides additional information on the pigment composition of our 4 Chl *a*/2 Pheo *a* RC complex. The absorption spectra of acetone extracts of both the 4 Chl *a* and 6 Chl *a* preparations were normalized at the Q_X transition of Pheo *a* at 535 nm and clearly show a reduction in the oscillator strength of the Q_Y band for our preparation, which is due to the lower number of Chl *a* molecules per RC, relative to the preparation made according to [13]. The cyt *b*-559 content was calculated from the dithionite-reduced minus ferricyanide-oxidized spectra and was ~1 cyt *b*-559 per 2 Pheo *a*.

The absorption spectra of the D1–D2–cyt *b*-559 complex containing 4 Chl *a*/2 Pheo *a* at 277 and 77 K in 0.1% dodecyl-*β*-D-maltoside are shown in Fig. 2. The spectra

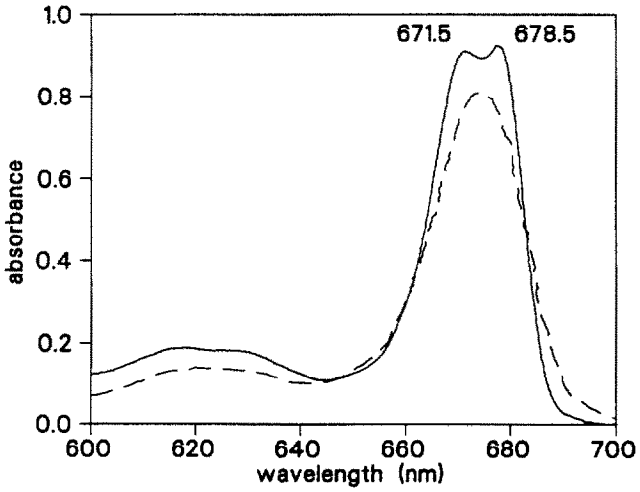

Fig. 2. Absorption spectra at room temperature (dashed) and 77 K (solid line) of the D1–D2–cyt *b*-559 complex containing 4 Chl *a*/2 Pheo *a*.

at 277 K exhibit a Q_Y band maximum at 675.5 ± 0.3 nm, well in accordance with values reported ealier [3,4,16,24]. *β*-carotene causes the absorption maximum at 482 ± 1 nm; the pheophytin Q_X band occurs at 542 ± 0.3 nm. The maximum at 416 ± 0.3 nm is due to the Soret bands of all chlorins and cytochrome (short-wavelength absorption maxima are not shown). The absorption spectra at low temperature resolved Q_Y maxima at 671.5 ± 0.3 and 678.5 ± 0.3 nm; the absorbance at 678.5 was higher than that of the 671.5 nm band in contrast to the 4 Chl *a*/2 Pheo *a* preparation reported by Montoya et al. [16].

SDS-PAGE (Fig. 3) revealed the purity of our RC preparation. It showed an electrophoretic profile consisting of four bands of apparent molecular weight of 65, 34, 31 and 9 kDa which correspond to the D1/D2 heterodimer, D2, D1 and the *α*-band of cytochrome *b*-559, respectively (Fig. 3A) [1,3,13,16]. The *β*-subunit of cyt *b*-559 of apparent molecular weight of 5 kDa could be resolved using 15–20% polyacrylamide gel (Fig. 3B). The sample was further characterized by its fluorescence emission and excitation spectra at 297 K and 77 K (Fig. 4). The fluorescence emission spectra at 297 and 77 K have maxima at 680 ± 2 nm and 683 ± 2 nm, respectively. Maxima were also observed at 730 ± 2 nm and 741 ± 2 nm, respectively. The absence of a shoulder or peak at 672 nm in the 77 K fluorescence measurements (Fig. 4) indicates that unconnected pigments do not contribute to the steady-state emission. Also, there is no indication of CP47 contamination, since no emission component around 690 nm could be observed [25]. A fluorescence excitation spectrum at 77 K was recorded in the range 500–720 nm for the emission at 740 nm. It showed a maximum at 674.5 ± 2 nm, indicating that the emission of 740 nm does not occur from excited states of P680 exclusively, but also from states that can be attributed to other pigment molecules, probably including Pheo *a*.

The photochemical activity of the 4 Chl *a*/2 Pheo *a* RC sample was checked by measuring the light-induced absorption change in the presence of sodium dithionite and methyl viologen (Fig. 5). A reversible bleaching with minima at 682 and 543 nm, associated with pheophytin reduction [1,3,15] was found in this experiment, indicating that charge separation between P680 and Pheo *a* takes place in our preparation.

In Fig. 6 the results of the flash-induced absorption difference measurements are summarized. Under anaerobic conditions the lifetime of the signals at 4°C was 1.0 ms. No indication of other millisecond decay components was found. The maximum bleaching was found to occur at 680 nm. The relative quantum yield at 77 K is about a factor of three higher than at 277 K, as can be derived from the initial slopes of the graphs of ΔA vs. flash energy.

These absorption changes can be attributed to the triplet state of P680, since lifetime, spectrum (not shown)

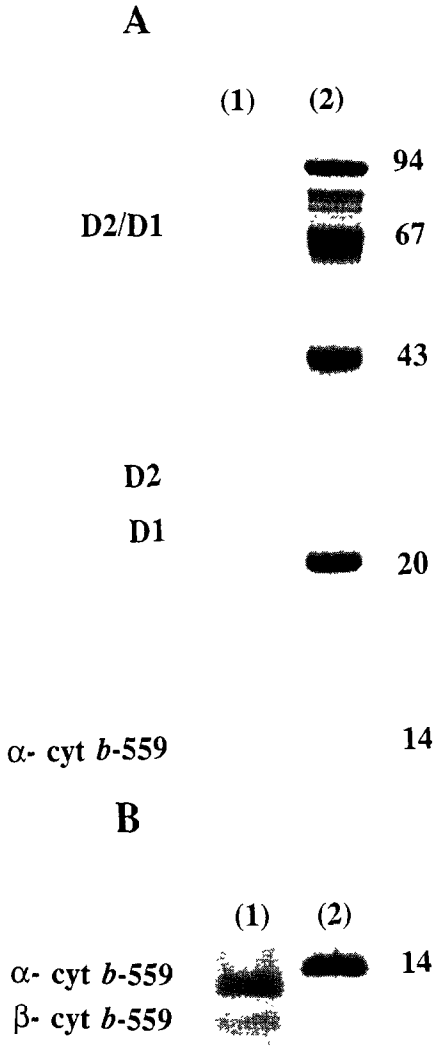

Fig. 3. SDS-PAGE carried out on a (A) 12.5% and (B) 15–20% polyacrylamide gel with 6 M urea. The protein bands were stained with Coomassie brilliant blue. (A) Lane 1, D1–D2–cyt *b*-559; lane 2, markers (94, 67, 43, 30, 20 and 14 kDa). (B) Lane 1, α- and β-subunit of cyt *b*-559; lane 2, marker (14 kDa).

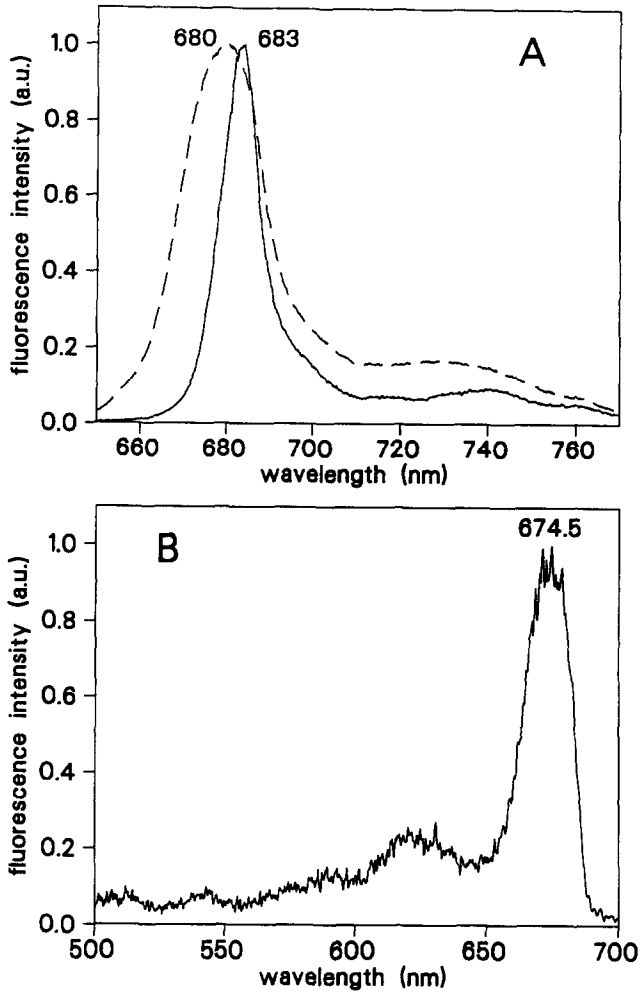

Fig. 4. (A) Fluorescence emission spectra at 297 K (dashed) and 77 K (solid line) from the D1–D2–cyt *b*-559 complex. Excitation wavelength 422 nm (emission bandwidth 2.5 nm FWHM). (B) Fluorescence excitation spectrum at 77 K for the emission detected at 740 nm with a bandwidth of 5 nm FWHM. Excitation bandwidth 3 nm.

and temperature dependence of the relative quantum yield agree very well with previous results [26]. We thus conclude that the triplet formation and decay parameters in our preparation are similar to those measured for other PS II RC preparations obtained by different methods.

4. Discussion

The chromophore composition of the D1–D2–cyt *b*-559 complex is still subject to debate. Recently, Montoya et al. [4,16] have reported that it is possible to obtain two different stoichiometries in the isolated PS II RC complex of the same biological material depending only on the detergent (i.e. Triton X-100) exposure time during the purification procedure. Both stoichiometries differ in the number of chlorophylls and β-carotene molecules. The short-time and long-time Triton-treated RCs thus obtained contain 6 Chl *a* and 4 Chl *a* per 2 Pheo *a*, respectively. Recently, several reports pointed out that the former ratio corresponds to the native D1–D2–cyt *b*-559 complex and new isolation methods have been developed to obtain this stoichiometry with high yield [13]. In contrast, the available procedures to prepare 4 Chl *a*/2 Pheo *a*, which basically follow the original method of Nanba and Satoh [1], used to give low yields.

In this paper we have described a new method to prepare 4 Chl *a*/2 Pheo *a* PS II RC with high yield. It combines the use of a strong anion-exchange column equilibrated with divalent cations (i.e. Q-Sepharose Fast-

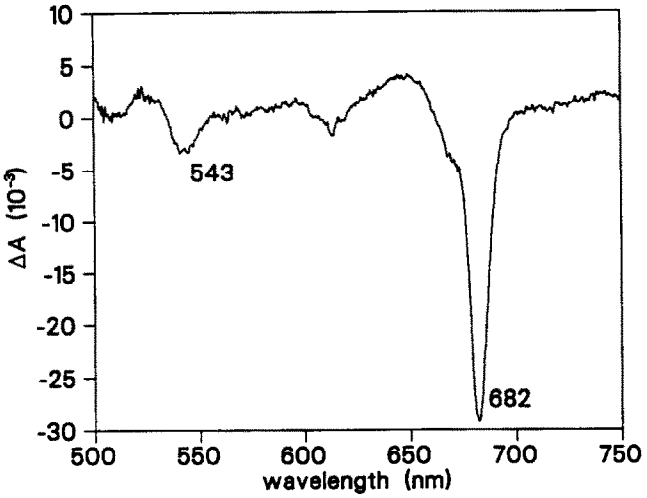

Fig. 5. Steady-state light-induced (light-on minus light-off) absorption changes at 277 K in the 500–750 nm spectral range. The sample absorption was 0.7 at 675.3 nm. Methyl viologen (1 μM) and 10 mM sodium dithionite were added.

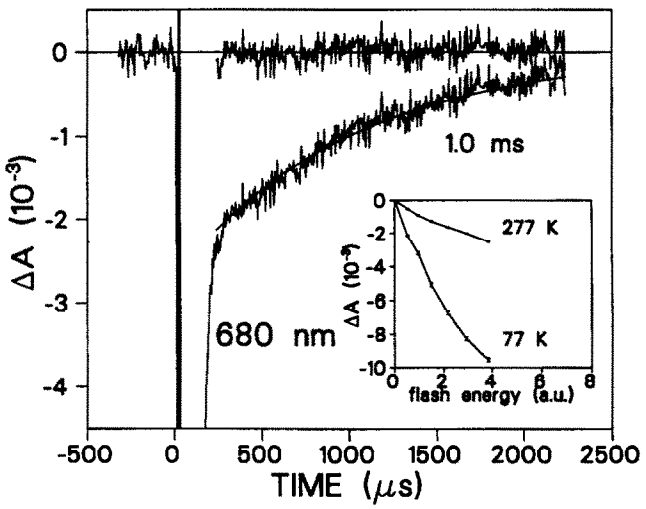

Fig. 6. Kinetics at 277 K of flash-induced absorption changes at 680 nm under anaerobic conditions. Fitting with a mono-exponential decay function from 250 μs onwards, with a lifetime (l/e) of 1.0 ms yields the residuals shown in the top curve. Inset: absorbance difference at 680 nm as a function of excitation energy, measured at 277 and 77 K.

Flow and $MgSO_4$) and a prolonged Triton X-100 treatment. This combination gave a 10 times higher yield than that described in [4]. The use of divalent cations in the purification step was essential for a rapid eluation of LHCII [13] and the capacity of the column helped to obtain a higher yield. As is the case with all ion-exchange materials, the capacity is dependent upon the number and accessibility of the charged groups. Q-Sepharose Fast-Flow ion exchanger is highly substituted and has a high capacity for proteins (180–250 μmol/ml). In contrast, a DEAE ion exchanger has a lower capacity (110–160 μmol/ml). The absorption and fluorescence spectra indicate that our D1–D2–cyt *b*-559 is comparable to those reported earlier [1–3] in most spectroscopic properties. It was furthermore fully active in charge separation as shown by reversible photoaccumulation of Pheo *a* (Fig. 5).

A selective bleaching around 680 nm has been observed upon oxidation of the primary electron donor P680 [24,25], ^{3}P680 formation [26] and reaction center inactivation [3,6].

We have tested the activity of our preparation in flash-induced triplet formation and found absorbance difference signals that showed the lifetime and spectral characteristics of ^{3}P680, as well as the expected temperature dependence of the quantum yield of triplet formation [26].

We conclude that we have developed a method for the isolation of a 'minimal' RC complex of photosystem II, that shows a photochemical activity which is quite similar to what is measured for other preparations, that have a higher Chl *a*/Phe *a* ratio.

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