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Disintegration and reconstitution of Photosystem II reaction center core complex. I. Preparation and characterization of three different types of subcomplex

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Disintegration of the Photosystem II (PS II) reaction center core complex (RC complex) from the spinach PS II by using mild detergents resulted in success of isolating three types of subcomplex (complexes 1–3), retaining their prosthetic groups and two pigments-binding subunit polypeptides with molecular masses of 47 kDa and 43 kDa. The polypeptide compositions of the three complexes isolated were 47 kDa/43 kDa/D1 cytochrome *b*-559 in complex 1, 47 kDa/D1/D2-cytochrome *b*-559 in complex 2 and D1/D2/cytochrome *b*-559 in complex 3. The complex 3 was confirmed to have four Chl *a*, two pheophytins *a*, one β-carotene and one plastoquinone A-9 (PQ_A) in molar ratio. These numbers were essentially the same as in the results reported by Nanba and Satoh on their D1/D2/cytochrome *b*-559 particle, except for PQ_A. All of the three subcomplexes isolated in the present work preserved one PQ_A at least. However, instead of the photoinduced charge separation between P-680 and Q_A the formation of the triplet state P-680 as a result of recombination of photoinduced P-680$^+$ and Pheo *a* was observed in the three preparations. The decay time of the triplet state was determined as 25 μs. Evidence suggesting that the reconstitution of Q_A function was performed in complex 1 and complex 2 with the thylakoid lipids and thylakoid lipids plus PQ_A, respectively, was obtained.

Abbreviations: PAGE, polyacrylamide gel electrophoresis; PS II, Photosystem II; LHCP II, light-harvesting chlorophyll *a*/*b* protein of Photosystem II; Chl, chlorophyll; P-680, reaction center of Photosystem II; Z, primary electron donor of Photosystem II; Q_A, primary stable electron acceptor of Photosystem II; Pheo *a*, pheophytin *a*; PQ_A, plastoquinone A-9; β-Car, β-carotene; FPLC, fast-protein liquid chromatography of Pharmacia system; Mono P, Pharmacia chromatofocusing column for FPLC; Mono Q, Pharmacia anion-exchange column for FPLC; HPLC, high-performance liquid chromatography; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; OGP, *n*-octyl-β-D-glucopyranoside; OTG, *n*-octyl-β-D-thioglucopyranoside; RC, reaction center.

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Introduction

It has been established that the minimum size of oxygen evolving Photosystem II complex is consisting of seven polypeptides [1–4], two of which (47 kDa and 43 kDa polypeptides) are chlorophyll-carrying polypeptides. Two distinct polypeptides with molecular masses of approx. 30 kDa, named as D1 and D2, tightly bind to each other and the former is identified as a secondary quinone acceptor (Q_B) binding polypeptide on the basis of its herbicides binding ability [5]. Cytochrome *b*-559 associated with the complex is believed to be consisting of two polypeptides [6], but it is not clear whether it is a homodimer of 10 kDa polypeptides [7] or a heterodimer of 10 kDa and 5

kDa polypeptides [8]. In addition to these intrinsic membrane polypeptides, and extrinsic polypeptide of 33 kDa which stabilizes manganese in water-splitting complex is bound to the oxygen-evolving complex. The attempts to identify the polypeptides forming the basis of the PS II photochemical reaction center have been done with less success until very recently. In early works, the 47 kDa polypeptide was proposed by several groups to be the site of P-680 and pheophytin *a* (Pheo *a*) binding based upon fluorescence [9], pigment composition and spectral change [10,11] and so on [12]. This was challenged by an alternative approach which compared the amino acid sequences of each subunit of the PS II complex with those of L and M subunits of reaction center of a purple photosynthetic bacterium whose structure had been determined by X-ray crystallographic analysis [13]. The sequence homology was found among D1, D2, L and M subunits, which suggested that the D1 and D2 complex would bind the PS II reaction center analogous to the L and M complex in the bacterial reaction center [14,15]. Recently, a conclusive evidence supporting the latter proposal has been provided by Nanba and Satoh [16]. They successfully isolated a complex consisting of D1 and D2 polypeptides and cytochrome *b*-559 from spinach PS II particles with 4–6 Chl *a*, 2 Pheo *a* and 1 β-Car molecules. This complex has photochemical activity to exhibit a reversible absorbance change attributed to the reduced Pheo *a* and to form a spin-polarized triplet at low temperature which is characteristic of all reaction centers [17]. It is of much interest that there is striking similarity in the pigment composition between the isolated complex and the purple bacterial reaction center. However, a quinone molecule which is present in the latter is not found in the former. The oxygen-evolving minimum size PS II complex contains two plastoquinone-9 (PQ_A). Therefore, the isolation procedure for the D1/D2/cytochrome *b*-559 (Nanba's particle) involving Triton X-100 treatment and DEAE chromatography causes to liberate PQ_A molecules from the complex. Such liberation might occur partly in other pigments including Chl *a*, Pheo *a* and β-Car involved in the reaction center during the preparation. In other words, it has not been ruled out completely that the pigment composition obtained in the Nanba's particle might not reflect the real feature of the pigment composition in PS II reaction center. Other distinct appraoch should be done to confirm that the pigment composition in the PS II reaction center is essentially equivalent to that of purple bacterial reaction center. In addition to this, the primary acceptor quinone (Q_A) to P-680 is not preserved in Nanba's particle and the reconstitution with it has never been reported. For now the 47 kDa polypeptide as well as the 43 kDa polypeptide is believed to serve only antenna functions, but some evidences indicating that only a preparation retaining the 47 kDa polypeptide will bind the quinone seem to suggest that it plays part in the quinone binding to the reaction center [17]. Aside the 47 kDa polypeptide, the 43 kDa polypeptide also may play a certain role other than antenna function. Our preliminary work on the interaction between the reaction center complex and the extrinsic 33 kDa polypeptide has revealed that only the D1/D2/cytochrome *b*-559 reaction center complex associated with the 43 kDa polypeptide will bind the 33 kDa polypeptide (unpublished results). Thus, although there seems little doubt that a D1/D2 complex forms the basis of the PS II reaction center, the roles of polypeptides other than D1 and D2 on the assembly of the PS II core complex and on the operation which leads to the stable charge separation in the reaction center are far from solved.

In this series of works, we disintegrated the PS II core complex prepared by the method of Ikeuchi et al. [2] into the D1/D2/cytochrome *b*-559 complex and the 47 and 43 kDa polypeptide subunits retaining their prosthetic groups by using a combination of *n*-octyl-β-D-glucopyranoside. (OGP) and *n*-octyl-β-D-thioglucopyranoside (OTG), with a view to determine the pigment distribution among the polypeptides in the PS II reaction center. Besides the D1/D2/cytochrome *b*-559 complex, we succeeded in isolating D1/D2/cytochrome *b*-559/47 kDa and D1/D2/cytochrome *b*-559/47 kDa/43 kDa complexes which are completely free from other polypeptides. Furthermore, we reconstituted the 47 kDa and/or 43 kDa polypeptides into the D1/D2/cytochrome *b*-559 complex with a help of thylakoid lipids, to reveal the roles of the 47 kDa and 43 kDa polypeptides.

In this paper, we report the preparations, pig-

ment compositions and photochemical activities of the isolated three subcomplexes and two individual polypeptides. In the subsequent papers, we will report the roles of the 47 kDa and 43 kDa polypeptides on the assembly and operation of the PS II complex by comparing the results obtained with the isolated subcomplexes and those with the reconstituted particles.

Materials and Methods

O_2-evolving PS II particles were prepared from fresh spinach leaves by the method of Kuwabara and Murata [18] and finally suspended in a solution of 0.2 M sucrose/20 mM NaCl/20 mM Mops (pH 7.0)/50 vol% glycerol to be stored in liquid N_2. Before use, the PS II preparation was washed with buffer solution A (0.2 M sucrose/20 mM NaCl/20 mM Mops (pH 7.0)). PS II particles depleted of three lumen-surface proteins, 18, 23 and 33 kDa proteins, were prepared by treating the PS II with 1 M $CaCl_2$ solution according to the method of Ono and Inoue [19]. To remove LHCP II from the $CaCl_2$-treated PS II particle, it was resuspended in a solution (60 mM OGP, 10 mM NaCl and 20 mM Mes-NaOH (pH 6.5)) at a chlorophyll (Chl) concentration of 1 mg/ml [2]. After incubation for 2 min at 0 °C, insoluble grey materials were removed from the suspension by centrifugation (20000 × *g*, 10 min, 3 °C), and the resulting green supernatant was set aside to be incubated at 0 °C for another 1 h and centrifugated (290000 × *g*, 3 °C, 2 h). The pellet was collected as PS II reaction center core complex (denoted as RC). Further disintegration of the RC was done by the following methods. Each RC was solubilized in solution B (20 mM OTG/25 mM Bistris (pH 7.1)) or in solution C (35 mM OGP, 20 mM NaCl, 20 mM Mes-NaOH (pH 6.5)) at a concentration of 0.4 mg Chl/ml. After incubation at 0 °C for 2 h with or without stirring, each sample was subjected to density gradient centrifugation or liquid phase column chromatography using a Pharmacia FPLC system.

Isolation of the subcomplexes with different polypeptide composition. RC sample solubilized in 5 ml of solution B or solution C was layered on a solution (28 ml) containing 35 mM OGP, 10 mM NaCl, 20 mM Mes-NaOH (pH 6.5) and 10–30% linear density gradient of sucrose and then centrifuged with a Hitachi RP70T angle rotor (140000 × *g*, 3 °C). After centrifugation for 14 h, two densed green bands were observed around sucrose concentration of 24% and 15%. The solution was fractionated from lower part to top (from fraction number 1 to number 19, 1.55 ml in each) leaving 3.5 ml of bottom part. A trace of green pellet was resuspended with the solution of the bottom part denoted as B. The polypeptide compositions and the prosthetic groups in each fraction were analyzed by SDS-PAGE and HPLC with a reverse phase column, respectively.

Isolation of 43 kDa and 47 kDa subunit polypeptides. 43 kDa and 47 kDa subunit polypeptides were isolated by using a Pharmacia FPLC system with an isoelectric chromatofocusing column (Mono P) and an anion-exchange column (Mono Q), respectively. RC was suspended in solution B at concentration of 0.4 mg Chl/ml and incubated at 0 °C for 2 h, followed by filtration through a Millex-GV (Millipore). A part of the resulting filtrate (2 ml) was loaded on a Mono P column preequilibriated with solution B (pH = 7.1) and eluted with solution containing 20 mM OTG and Polybuffer 74 (Pharmacia) diluted by a factor of 10 at pH 4.0. The other part of the filtrate (2 ml) was loaded on a Mono Q column preequilibrated with solution B and eluted with solution B changing NaCl concentration from 0 to 0.3 M.

Isolation of total lipids and plastoquinone A-9 (PQ_A). Thylakoid total lipids were isolated from the broken thylakoids as described in Ref. 20. PQ_A was extracted from PS II particles and fractionated roughly by a column chromatography on an alumina column according to the method of Barr and Crane [21]. This crude PQ_A was purified by a HPLC with a reverse-phase column (Waters, μ-Bondapak C_{18}). A mixed solvent (methanol/ethanol, 65 : 35, v/v) was used as the eluent.

Reconstitution of the three subcomplexes with lipids and/or PQ_A. The purified total lipids or the mixture of the lipids and PQ_A were dried under reduced pressure from chloroform-ethanol solution and comicellized with 60 mM OGP solution. To each of the three subcomplex preparations was added either the comicellized lipids (Chl *a*/lipids, 1 : 5 in molar ratio) or lipids plus PQ_A(PQ_A/Pheo

a, 4:1) solution. After adjusting, the concentrations of Chl *a* and OGP to 0.15 mg/ml and 35 mM, respectively, the mixture was incubated for 30 min in ice, dialyzed against a solution of 50 mM Mes, 10 mM NaCl and 5 mM $CaCl_2$ at pH 6.0 for 4 h in ice, changing the buffer solution more than twice. The dialyzed solution was diluted 10-fold with the same buffer, and finally centrifuged at 40000 × *g* for 30 min at 4° C. The resulting pellet was resuspended in solution A and submitted to the flash photolysis experiment.

SDS-PAGE. SDS-PAGE was carried out according to Ref. 22 with the modification that 6 M urea was added in stacking and separating gels. A uniform separating gel of 10% acrylamide was used. Sample solution was mixed with a solution (2.5% SDS, 125 mM dithiothreitol, 10 mM NaCl, 20 mM Mes-NaOH (pH 6.5) and 10% sucrose) of equivalent volume, and electrophoresed at room temperature with a constant current of 4 mA. Gels were stained with silver [23] or Coomassie brilliant blue R250. A German DCD-16 digital computing densitometer was used to analyze the stained density of each band on the gel.

Analyusis of the prosthetic groups (Chl a, Pheo a, β-Car and PQ_A). The prosthetic groups contained in each preparation were analyzed by the method of Eskins and Dutton [24] with modification. 200 μl sample was loaded to a Waters Associates Sep-Pak cartridge (reverse phase, μ-Bondapak C_{18}) activated with 4 ml 70% methanol. After washing with 2 ml 70% methanol, the compounds absorbed on the cartridge were eluted out completely with a mixed solvent (ethanol/ethylether: 1:1, v/v, 4 ml) and collected. The whole effluent was dried immediately with a rotory evapolator and dissolved in 400 μl ethanol to be injected into a HPLC (Waters, 6000A, reverse-phase column of μ-Bondapak C_{18}). Elution of the prosthetic groups was monitored at 270 nm. The amounts of separated pigments and PQ_A were determined spectrophotometrically, using molar extinction coefficients, 51100 at 664 nm for Pheo *a* [25], 138000 at 448 nm for β-carotene [26], 86300 at 660 nm for Chl *a* [27] and the oxidized-minus-reduced molar extinction coefficient, 15000 at 255 nm for PQ_A [28].

Measurements of flash induced absorbance changes at 680 nm and 830 nm. Recovery of P-680 and relaxation of $P\text{-}680^+$ after flash illumination were measured by monitoring the absorbance change at 680 nm and 830 nm, respectively, with a single-beam flash spectrophotometer basically similar to the one described in Ref. 29. The measuring light was provided by a halogen lamp through an interference filter at 680 nm or 830 nm. Passing through a couple of condensing lenses, the measuring beam was focused on 2 cm behind the sample cuvette. A slit was placed 0.5 cm before the cuvette. With another condensing lens the measuring beam which passed through the sample solution was focused again on the entrance slit of a monochromator (JASCO, CT-25A) set at 680 or 830 nm and detected by a photomultiplier module (Hamamatsu, R-374). 1025 signals were amplified and digitized by a transient recorder (Iwatsu, DM-703 Digital Memory) and accumulated by a NEC 9801-M personal computer. Time resolution of the detecting system was limited to about 1 μs in order to suppress interference from fluorescence. Flash excitation was performed with pulses (pulse width, 7 ns; intensity, 30 mJ per pulse) from a Q-swiched frequency-doubled Nd:YAG laser (NEC SL 120D). The measurements were carried out in a 5 mm light pass-length of cuvette. Before the flash experiments each sample solution was kept in darkness on ice. After 5 min of additional dark adaptation in the cuvette at 25° C, the sample was illuminated by a train of laser flashes at 10 Hz and the absorbance change at 680 nm or 830 nm was recorded for each flash and accumulated.

Results and Discussion

Polypeptide compositions of the isolated particles

Fig. 1 shows an example of the results on the polypeptide compositions and the amounts of four prosthetic groups contained in each fraction after sucrose density gradient centrifugation. This experiment was carried out with the RC solubilized with solution B without stirring. Judging from the silver-stained SDS-PAGE shown in the upper figure, the 47 kDa, 43 kDa, D1, D2 and cytochrome *b*-559 are dominant in the RC obtained by the present method, but other several polypeptide components still remain to some extent, including major LHCPs with molecular masses of 27–29

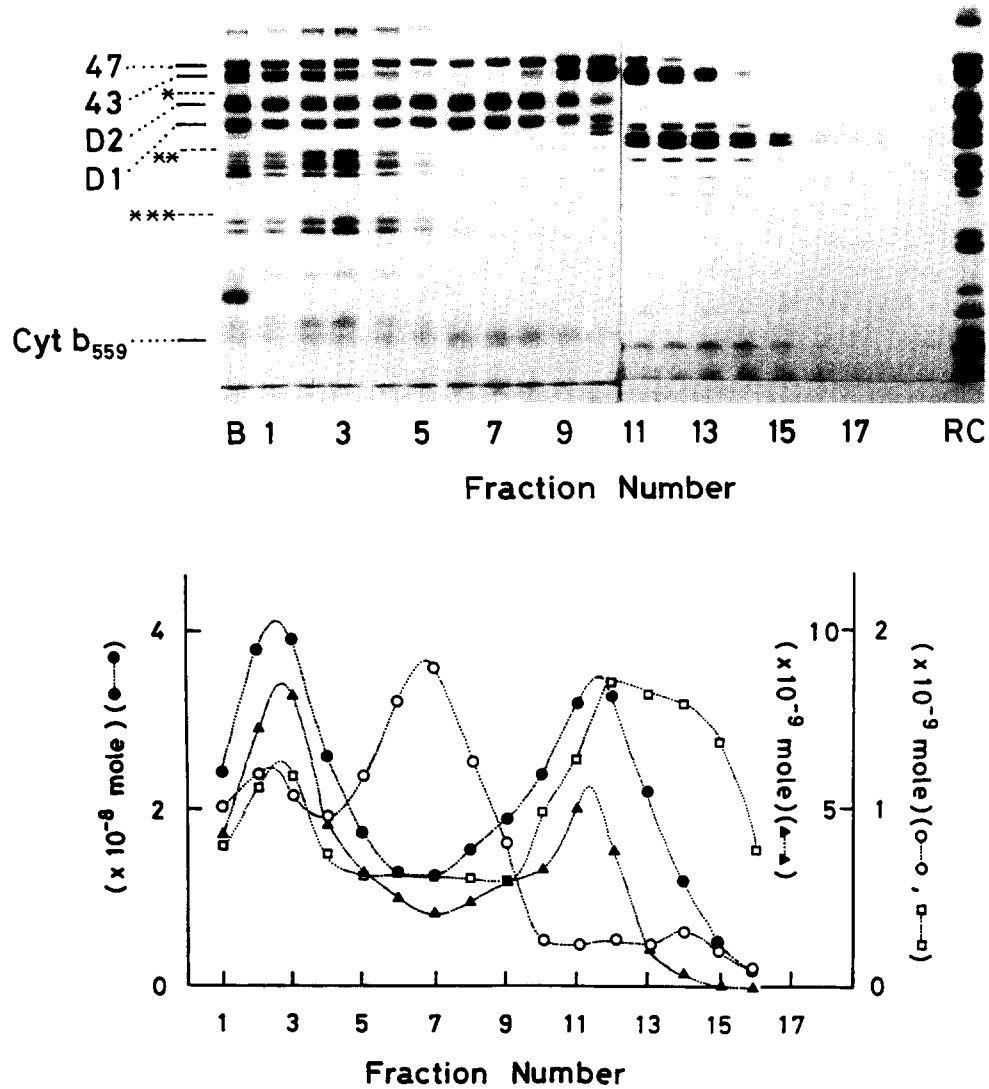


Fig. 1. Polypeptide compoisitions and amounts of four prosthetic groups (Chl *a*, Pheo *a*, β-Car, PQ_A) in each fraction after sucrose density gradient centrifugation. In this experiment, the RC was solubilized in solution B at a concentration of 0.4 mg Chl per ml and incubated at 0 °C for 2 h without stirring and subjected to the centrifugation. The upper figure shows the polypeptide compositions in fractions B–19 and RC on the silver stained gel, where * ------, ** ------ and *** ------ indicate the positions of the 33 kDa, 23 kDa and 18 kDa extrinsic polypeptides, respectively. The lower figure represents the amounts of four prosthetic groups in the corresponding fractions. Chl *a* (●), Pheo *a* (○), β-Car (△), PQ_A (□).

kDa and minor LHCPs of 25–15 kDa. After treatment with 20 mM OTG followed by the sucrose gradient centrifugation, the major LHCPs were liberated from the complex and recovered in fractions 10–16. In addition to these, a large portion of the 43 kDa polypeptide and a part of the 47 kDa polypeptide were liberated and recovered in fractions 9–13 and fractions 8–12, respectively. While the other components were not liberated by these treatments. These results suggested a possibility to isolate several types of complex with different polypeptide compositions. The lower graph exhibits the profiles of four prosthetic groups as a function of fraction number. It had been confirmed that when free pigments comicellized with OGP and/or OTG were present in the

centrifuged sample, they appeared at around the fraction 14 under the present centrifugal conditions. Considerable amount of PQ_A was observed in this region with a shoulder at the fraction 14, indicating that some portions of PQ_A were liberated from the native sites during the disintegration process of the RC. In contrast with PQ_A, the patterns of Chl *a*, Pheo *a* and β-Car have no peak in this region, except for a very small peak at the fraction 14 for Pheo *a*. Therefore, we concluded that these three pigments remain completely to the corresponding native sites even after the above detergent treatments. Under these situations, quantitative comparison of the polypeptide composition and the amount of three pigments in each fraction made it possible to estimate the numbers of the pigments bound on the different types of subcomplex. At first, an attention was given on Pheo *a*, because it was considered to exist in thylakoid membranes only as the primary electron acceptor of the PS II reaction center. The profile of Phe *a* exhibited two peaks at fractions 2 and 7 accompanied by peaks of D1, D2 and cytochrome *b*-559 subunit polypeptides. The content of Pheo *a* in each fraction was essentially proportional to those of D1, D2 and cytochrome *b*-559 estimated by the silver-stained density on the band corresponding to each polypeptide (data are not shown), but did not show any correlation to the contents of other subunit polypeptides including the 47 kDa and 43 kDa polypeptides. A possibility that these Pheo *a* molecules originate from the decomposition product of Chl *a* during the disintegration and/or analysis processes rather than the native Pheo *a* in the reaction center was ruled out, because the summation of the content of Pheo *a* over every fraction after the centrifugation was equal, in experimental error, to the amount of Pheo *a* in the starting RC before solubilization with 20 mM OTG.

Then, we attempted to reveal how many Chl *a*, Pheo *a*, β-Car and PQ_A are bound to each subunit and subcomplex, and what is the minimum size complex which maintains the primary charge separation activity of the PS II. For these purposes, we prepared three different types of subcomplex and isolated two subunits, 47 kDa and 43 kDa with retaining the pigments. Fig. 2 shows the polypeptide compositions in the preparations of the three subcomplexes and two isolated subunits obtained in the present work. To prepare the complex composed of the 47 kDa, 43 kDa, D1, D2 and cytochrome *b*-559 denoted as complex 1, the RC was solubilized in solution C, incubated at 0 °C for 1 h without stirring and centrifuged in 10–30% sucrose gradient in the presence of 35 mM OGP. Complex 1 was obtained in fraction 6. The complex of the 47 kDa, D1, D2 and cytochrome *b*-559, complex 2, was prepared by essentially the same procedure as described in the legend of Fig. 1. For the preparation of the complex composed of D1, D2 and cytochrome *b*-559, complex 3, the RC was solubilized in solution B and

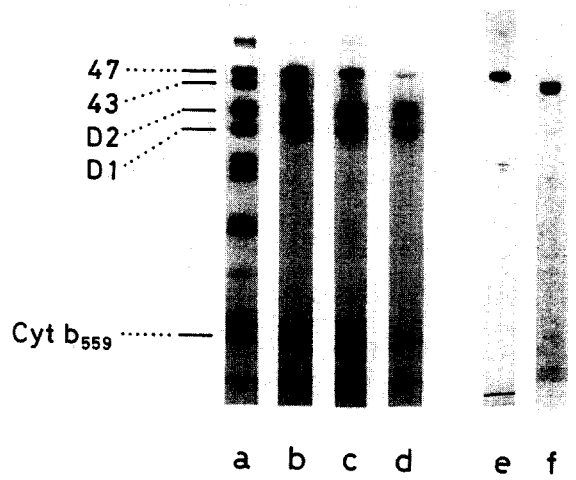
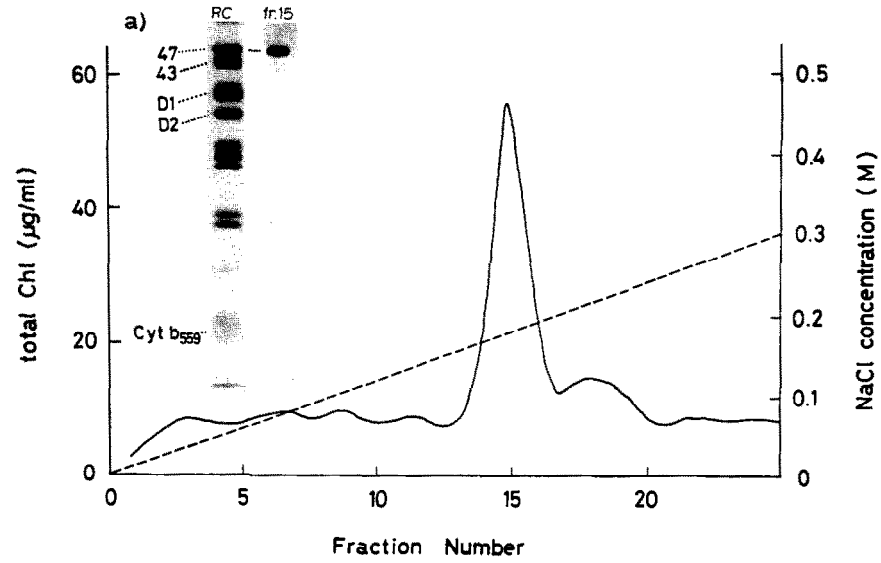
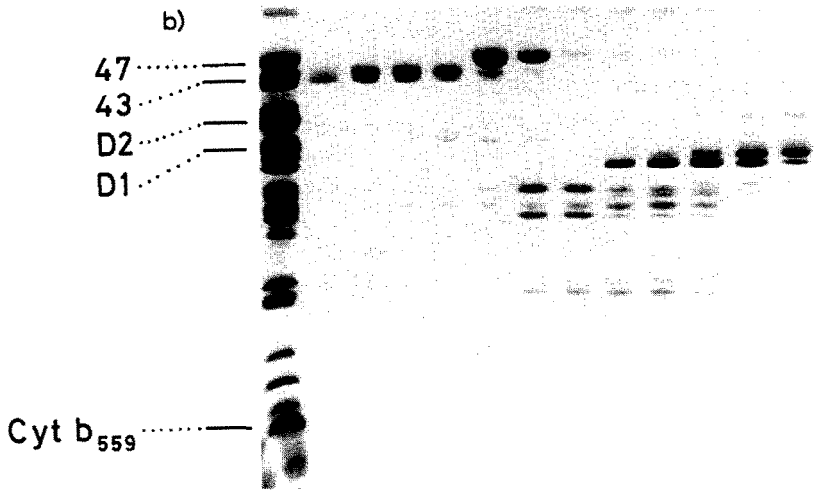
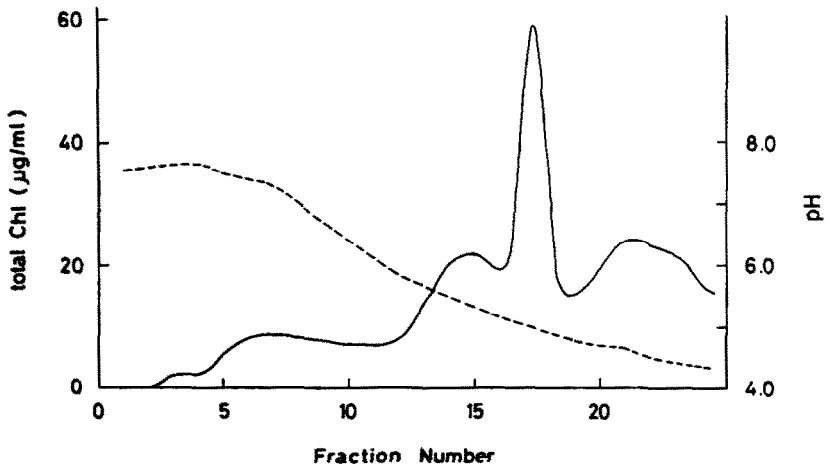


Fig. 2. Polypeptide compositions in the preparations of three subcomplexes and two isolated subunits on silver stained SDS-PAGE (10% homogeneous slab gel). (a): RC, (b): complex 1, (c): complex 2, (d): complex 3, (e): 47 kDa polypeptide, (f): 43 kDa polypeptide.

Fig. 3. (a). Isolation of 47 kDa polypeptide from the RC complex by using an anion exchange column (Pharmacia, Mono Q). The loaded sample was eluted with solution B additionally containing NaCl of which concentration was varied from 0 to 0.3 M. The polypeptide was isolated in fraction 15. The purity was confirmed by a SDS-PAGE with silver staining. (b). Isolation of 43 kDa polypeptide from the RC complex by using an isoelectric chromatofocusing column (Pharmacia Mono P). Polypeptide compositions in each fraction were determined by silver stained SDS-PAGE. 43 kDa polypeptide was isolated in fractions 13–15 (pH range: 5.5–6.0).


a)
RC
fr.15
47
43
D1
D2
Cyt b559
total Chl (µg/ml)
NaCl concentration (M)
Fraction Number


b)
47
43
D2
D1
Cyt b559
RC 13 15 17 19 21 23


total Chl (µg/ml)
pH
Fraction Number

incubated at 0 °C for 2 h under stirring, contrary to the case of the complex 2 preparation. Then the solution was centrifuged under the same condition as for complex 1. Fraction 7 contained complex 3 dominantly, but unfortunately it was contaminated with the complex 2 and/or the liberated 47 kDa subunit polypeptide to some extent as seen in Fig. 2 (lane d). In addition to these three subcomplexes, the 47 kDa and 43 kDa subunit polypeptides were isolated by means of a Pharmacia FPLC system using an anion-exchange column and a chromatofocusing column, respectively, in the presence of 20 mM OTG. Elution profile of each column chromatography is shown in Fig. 3a and 3b, respectively.

Pigments compositions in the isolated particles

Analysis of the prosthetic groups for the two isolated subunits and three subcomplexes together with the RC, PS II particles and the D1/D2/cytochrome *b*-559 complex prepared by Nanba and Satoh's method [16] (Nanba's particle) was carried out by using of a Waters HPLC system with a reverse phase column. The isolated 47 kDa subunit contained Chl *a* and β-Car at molar ratio of $(10 \pm 1):1$, but not Pheo *a* and PQ_A at all. While, the isolated 43 kDa subunit bound Chl *a* and β-Car at molar ratio of $(9 \pm 1):1$ and a trace of Pheo *a*. This Pheo *a* presumably originated from the decomposition product of Chl *a* during isolation, because the protein was eluted out at the range pH 5.5–6.0 from the chromatofocusing column. PQ_A was not detected in the isolated 43 kDa subunit. Results obtained for three subcomplexes are summarized in Table I in the form of molar ratio on base of two Pheo *a* molecules and compared with the corresponding data of the RC and Nanba's particle. As mentioned before, preparation of complex 3 obtained by the density gradient centrifugation was contaminated with complex 2 and/or the liberated 47 kDa polypeptide to some extent which leads over estimation on Chl *a* and β-Car in complex 3. The error which originates from the contaminated 47 kDa polypeptide was eliminated as follows. SDS-PAGE was carried out with the complex 3 preparation and the isolated 47-kDa polypeptide on one gel plate with several lanes, changing the loading amount for the latter and the Coomassie brilliant blue stained density of the 47 kDa band on each lane was measured. Fig. 4 shows the plots of the amount of Chl *a* loaded on each lane against the stained density of the band corresponding to 47 kDa. With the data obtained for the isolated 47 kDa preparation, the plots followed a straight line through the origin. This line was used as a working curve for estimating the amount of Chl *a* attributed to the 47 kDa polypeptide. The value of Chl *a* given for complex 3 in Table I corresponds to the net Chl *a* involved in complex 3 itself, i.e., total Chl *a* involved in the complex 3 preparation minus Chl *a* attributed to the 47 kDa polypeptide contaminated in the preparation. Essentially the same procedure was used for β-Car but not for PQ_A and Pheo *a*, because of the absence of the latter two pigments in the isolated 47 kDa preparation. The relative amount of Chl *a* to Pheo *a* decreased with the stepwise removal of the 43 kDa and 47 kDa subunits from

TABLE I

ANALYSIS OF THE PROSTHETIC GROUPS FOR THE REACTION CENTER COMPLEX, THREE SUBCOMPLEXS AND (D1/D2/CYTOCHROME *b*-559) COMPLEX PREPARED BY NANBA AND SATOH'S METHOD

Sample	Chl *a*	Pheo *a*	β-Car	PQ_A
Reaction center complex	73.4	2	15.6	2.2
Complex 1 (47/43/D1/D2/cytochrome *b*-559)	28.2	2	6.4	1.4
Complex 2 (47/D1/D2/cytochrome *b*-559)	17.0	2	3.4	0.8
Complex 3 (D1/D2/cytochrome *b*-559)	3.8±0.4	2	0.8±0.2	0.8±0.2
Nanba's particle (D1/D2/cytochrome *b*-559)	4.2	2	1.5	0

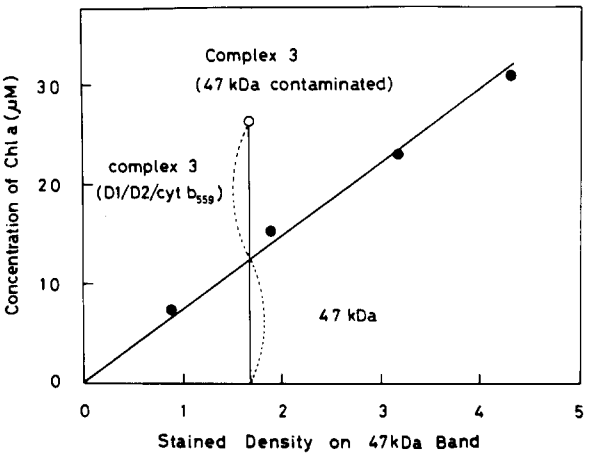


Fig. 4. Plots of Chl *a* concentrations against stained densities on the 47 kDa polypeptide band. ●, the isolated 47 kDa polypeptide on one gel plate changing the loaded amount of the polypeptide. ○, complex 3 preparation contaminated with 47 kDa polypeptide. The amount of Chl *a* in complex 3 was estimated by subtracting the amount of Chl *a* attributed to the 47 kDa polypeptide from the total Chl *a* involved in the complex 3 preparation. Polypeptides were stained with Coomassie Brilliant Blue R250.

the complex and finally reached about four molecules to two Pheo *a* molecules in complex 3. Similarly the molar ratios of PQ_A and β-Car to Pheo *a* became about one molecule to two Pheo *a*, respectively. These results coincide with the results obtained with the Nanba's particle except for PQ_A [15]. The latter has no PQ_A, whereas the former is associated with one PQ_A to two Pheo *a*.

Photochemistry of the three subcomplexes

In order to investigate the photochemistry of the three complexes prepared in the present work, we measured the transient absorbance change at 680 nm ($-\Delta A_{680}$) and 830 nm (ΔA_{830}) with the three subcomplexes and compared with those of the isolated 47 kDa and 43 kDa polypeptide preparations and the Nanba's particle. In the particles with intact water-splitting system, the photooxidation and rereduction of P-680 occur in the picosecond and nanosecond time scales, respectively. Both the processes cannot be detected by the present instrument with a time resolution limited to about 1 μs. However, if the electron transfer on the donor side of PS II is inhibited and the acceptor side is preserved at least until the Q_A site, it is possible to observe the signal for the photoinduced P-680$^+$ because its decay kinetics slow down to a microsecond time scale [30,31]. Therefore, in this case, the transient absorbance changes at 680 nm and 830 nm provide direct monitors of the photobleach and recovery of the

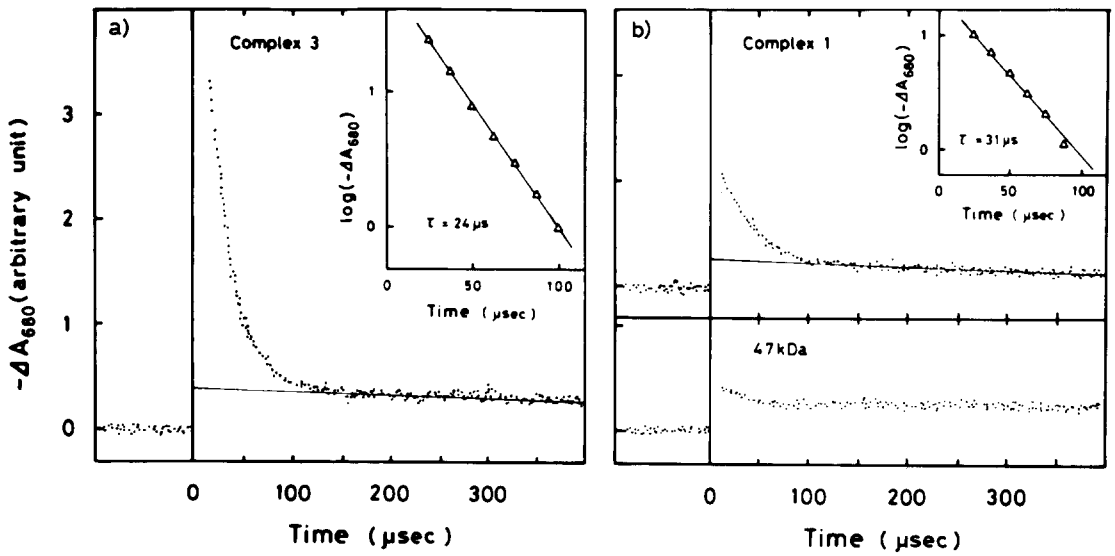


Fig. 5. Traces of photoinduced transient absorbance change at 680 nm in the preparations of complex 1, complex 3 and 47 kDa polypeptide. Temperature, 25 ± 1° C. Samples were suspended in solution A at concentration of 26 μg Chl/ml with 0.1 mM $K_3Fe(CN)_6$, respectively. Insets represent the plots of $\log(-\Delta A_{680})$ against time (μs).

P-680 and of the formation and decay of the P-680$^+$, respectively. Fig. 5 shows typical traces of $-\Delta A_{680}$ induced by repetitive laser flashes in complex 1, complex 3 and the 47 kDa subunit preparations in the presence of 0.1 mM $K_3[Fe(CN)_6]$. In complex 1 and complex 3, a rapid decrease in A_{680} within 1 μs and a recovery consisting of two phases are observed. The relaxation time of the rapid component which follows a single exponential curve (see Fig. 5) was about 25 μs and that of the slow component was in the millisecond range in each complex. Essentially the same signal was observed in the complex 2 and in Nanba's D1/D2/cytochrome *b*-559 particle but not in the isolated 47 kDa and 43 kDa subunit preparations. The initial amplitudes of the absorbance change of the microsecond component, $-\Delta A^{init}_{680,\mu}$ of the preparations of complex 2 and 3 were larger than that of complex 1 by a factor of 2 and 4, respectively, when the Chl *a* concentration in the samples were equal to each other. These results suggest that the reversible absorbance change at 680 nm upon flash illumination is associated with the photochemical reaction center locating on the D1/D2/cytochrome *b*-559 complex. This presumption was confirmed by the results obtained in the same experiment with the fractions 1–11 prepared by the same procedure for the experiment shown in Fig. 1. Fig. 6 shows the plots of $-\Delta A^{init}_{680,\mu}$ and the relaxation time of the microsecond component, τ, against the Pheo *a*

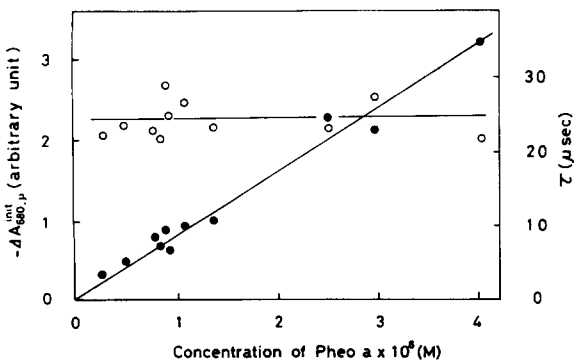


Fig. 6. Pheo *a* concentration dependence of the initial amplitude of absorbance change, $-\Delta A^{init}_{680,\mu}$, (●) and the relaxation time, τ, (○) of the microsecond component. Data were obtained under similar experimental conditions as those of Fig. 5 with the fractions 1–11 prepared by the same procedure for the experiment shown in Fig. 1.

concentration with the data obtained at a Chl *a* concentration of 26 μg/ml in the presence of 0.1 mM $K_3[Fe(CN)_6]$. $-\Delta A^{init}_{680,\mu}$ was proportional to the Pheo *a* concentration, while τ was about 25 μs independent of the fraction.

The transient absorbance change at 830 nm upon flash illumination was also examined and compared with the corresponding data obtained at 680 nm. Typical results are illustrated in Fig. 7 together with the results obtained in the Tris-treated PS II particles. In the case of the PS II of which the donor side was inhibited by Tris-treatment, a rapid increase in the absorbance at 830 nm was followed by the decay with time constant of about 160 μs, indicating the formation and rereduction of P-680$^+$. This decay kinetics are identical to that of the absorbance change transient at 680 nm in the depicted time range within an experimental error. Contrary to this case, the microsecond absorbance change at 680 nm upon flash illumination observed in the three complexes and the Nanba's particle was not accompanied by the absorbance change at 830 nm. The lack of the absorbance change at 830 nm in the complexes seems to suggest that the photobleaching of the absorbance at 680 nm and its microsecond recovery occur through a pathway other than formation of P-680$^+$. The PS II reaction center has been characterized by formation of the triplet state upon illumination at low temperature. This triplet state has been believed to arise from a reaction center Chl *a* as a result of recombination of P-680$^+$ and Pheo a$^-$ pair because of the inhibition of the forward electron transfer from Pheo a^- to Q_A at low temperature [17]. Therefore, if Q_A does not function in a reaction center, one may expect to observe the signals corresponding to the formation of the triplet state upon flash illumination even at room temperature. So it seems reasonable to conclude that the flash-induced microsecond absorbance change at 680 nm observed in the three subcomplexes and Nanba's particles is attributed to the formation of the triplet state P-680 as a result of recombination of photoinduced P-680$^+$ and Pheo a^- which occurs within the time resolution of our instrument. Consequently, it is considered that in the above four particles Q_A is lost or its function is inhibited. In that case, the relaxation time of the P-680 triplet state to the

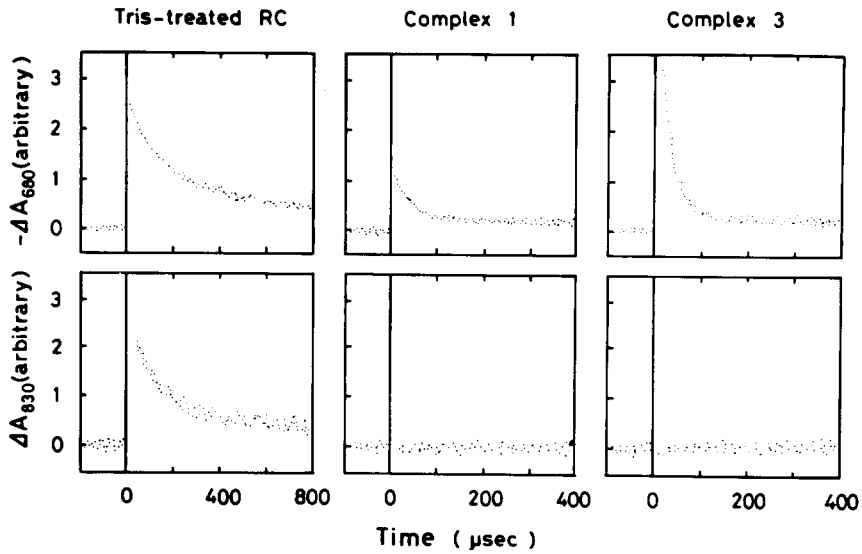


Fig. 7. Traces of transient absorbance changes at 680 nm and 830 nm induced by flash illumination in the preparations of complex 1 and complex 3 together with the Tris-treated PS II particles. Temperature, 25 ± 1 °C. Samples were suspended in solution A at concentrations of 50 μg Chl/ml for $-\Delta A_{680}$ and 100 μg Chl/ml for ΔA_{830} with 0.1 mM $K_3Fe(CN)_6$, respectively.

ground state is 25 μs. The proportionality between $-\Delta A^{init}_{680,\mu}$ and the amount of Pheo *a* with the data shown in Fig. 6 and those obtained for the three subcomples demonstrates that removal of the 47 and 43 kDa polypeptides by the present method does not result in depression of the yield of the triplet state formation in the reaction center. Thus, the D1/D2/cytochrome *b*-559 complex isolated from the RC without loss of Chl *a*, Pheo *a* and β-Car (complex 3) is fully active on the basis of the formation of the triplet state through recombination of P-680$^+$ and Pheo a^-. And its pigment composition is almost identical to that in purple bacterial center. These results strongly support the proposal that the assembly of the pigments as well as the polypeptides in PS II raction center is essentially similar to that of the purple bacterial reaction center.

Stability of complex 3

We compared the stability of complex 3 with Nanba's particle by measuring $-\Delta A^{init}_{680,\mu}$ after incubation for given periods in the dark at 0 °C and 25 °C. Fig. 8 indicates that as long as stored at 0 °C in the dark, the signal intensity in both the preparations did not change at all at least 24 h, but when incubated at 25 °C in the dark, the signal intensity rapidly decreased in the Nanba's particle, while it was held unchanged in the complex 3 preparation at least for 4 h.

Recovery of long life P-680$^+$ (τ = 130 μs) with the lipids and/or PQ_A

Contrary to Nanba's particle, the three subcomplexes isolated in the present work preserve one PQ_A at least. Nevertheless, the stable charge

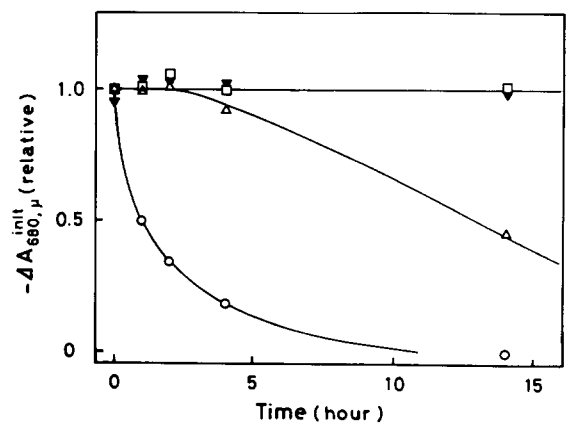


Fig. 8. Comparison of the stability to form the triplet state between complex 3 and Nanba's particle. ▼, complex 3 at 0 °C; □, Nanba's particle at 0 °C; △, complex 3 at 25 °C; ○, Nanba's particles at 25 °C.

separation to form long life P-680$^+$ was not observed in all of the subcomplexes. It had been found that the removal of lipids from the PS II complex by extraction with cholate detergent reversibly reduces the O_2-evolution rate [20] and this reduction is mainly due to the decrease in the stability of charge separation between P-680 and Q_A [32]. A large portion of lipids as well as PQ_A in the RC complex must be extracted during the preparation of the three subcomplexes, and this might be responsible for the lack of stable charge separation. In order to investigate the lipids and PQ_A effects on the photochemistry of the three subcomplexes we measured the transient absorbance change at 680 nm and 830 nm in the reconstituted complexes with the purified lipids and with the lipids and PQ_A. Fig. 9 shows typical traces of A_{680} induced by repetitive laser flashes in the reconstituted complexes. The reconstitution with the lipids significantly recovers the 130 μs component of the relaxation kinetics in complex 1 at the expense of the 25 μs component, but in complex 2 as well as complex 3 the lipids do not show a significant effect on the relaxation kinetics. When the reconstitution was carried out with PQ_A together with the lipids, however, the 130 μs component appears in complex 2 associated with the slower kinetics in the millisecond region. The addition of PQ_A to the reconstitution medium for complex 1 enhances the signal amplitude of the 130 μs component (Fig. 9(a), C). Contrary to the 25 μs component in $-\Delta A_{680}$, the 130 μs component recovered in the reconstituted complexes was accompanied by the recovery of the transient absorbance change at 830 nm (data not shown). This seems to indicate that stable charge separation to form P-680$^+$ actually takes place in the reconstituted complexes. The recovery of the 130 μs component in A_{680} was also observed in complex 3 reconstituted with PQ_A and lipids, but its signal intensity was smaller than that in the reconstituted complex 2 under same Pheo *a* concentration. However, reproducibility of the signal intensity in complex 3 was too poor to make a conclusion about the involvement of the 47 kDa polypeptide to the Q_A binding site only by comparing the data obtained with the two preparations. We confine ourselves at the moment with the presentation of

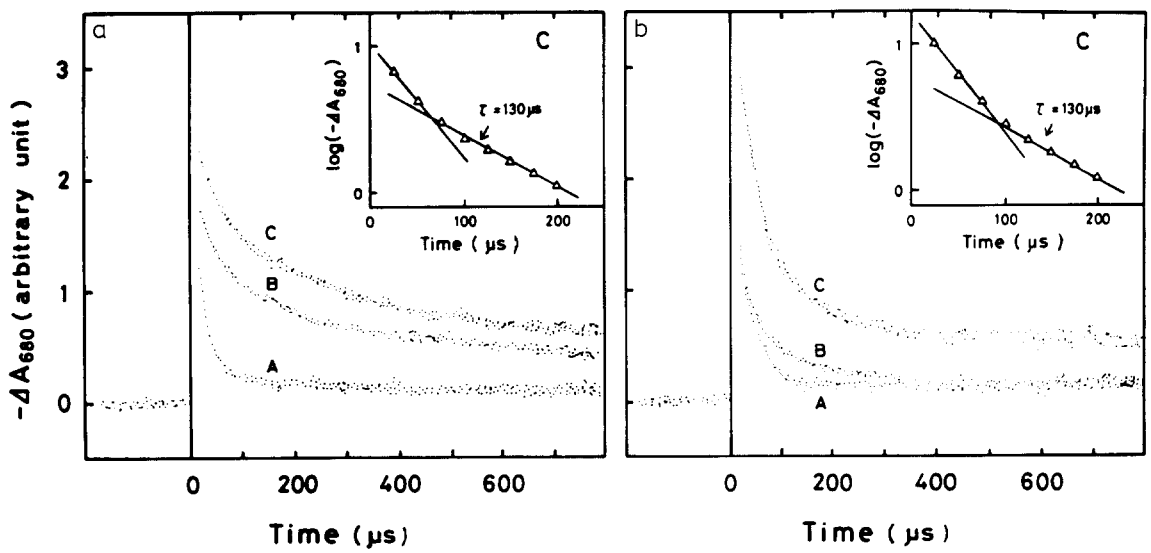


Fig. 9. Traces of photoinduced transient absorbance change at 680 nm in the reconstituted complexes with the thylakoid lipids and with the lipids and PQ_A. (a): A, complex 1 (control); B, reconstituted complex 1 (lipids), lipids/Chl *a* ratio, 3.0:1.0 (w/w); C, reconstituted complex 1 (lipids, PQ_A), lipids/Chl *a* ratio, 3.0:1.0 (w/w), PQ_A/Pheo *a* ratio, 4.0:2.0 (mol/mol). (b): A, complex 2 (control); B, reconstituted complex 2 (lipids), lipids/Chl *a* ratio, 5.0:1.0 (w/w); C, reconstituted complex 2 (lipids, PQ_A), lipids/Chl *a* ratio, 5.0:1.0, PQ_A/Pheo *a* ratio, 8.0:2.0. Flash experiments were carried out with the samples suspended in solution A at the concentration of 50 μg Chl *a*/ml with 0.1 mM $K_3Fe(CN)_6$, respectively. Insets represent the plots of $\log(-\Delta A_{680})$ against time (μs) with the corresponding data.

the results which demonstrate that the Q_A function depleted in the complex 2(D1/D2/cytochrome *b*-559/47 kDa) is able to be restored by the addition of PQ_A together with the thylakoid lipids.

In subsequent papers, we will report the reconstitution of the 47 kDa and/or 43 kDa polypeptides into the D1/D2/cytochrome *b*-559 complex with a help of the thylakoid lipids to reveal the roles of these two subunits on the assembly of Q_A and the extrinsic 33 kDa polypeptide in the PS II complex.

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