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Further characterization of the two Photosystem II reaction center complex preparations from the thermophilic cyanobacterium *Synechococcus* sp.

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Photochemical and chemical properties of two Photosystem II reaction center complexes isolated from the thermophilic cyanobacterium *Synechococcus* sp. were examined. (1) The intact reaction center complexes contain each one of photoreducible pheophytin, secondary electron acceptor (Q_A) and cytochrome *b*-559 per 32–46 chlorophyll *a* molecules. (2) The reaction center complexes which lack the chlorophyll-binding 40 kDa polypeptide (CP2-b) showed photoaccumulation of reduced pheophytin and photoreduction of Q_A, indicating that the complexes can carry out not only the primary-charge separation, but also the stabilization of the separated charges. The contents of pheophytin, Q_A and cytochrome *b*-559 were, however, considerably reduced in CP2-b. (3) The two complexes contained very small amounts of manganese. (4) CP2-b was partially deprived of the small polypeptides: the ratios of the peak areas (corrected for molecular weight) of the 47/40/31 plus 28/9 kDa polypeptide bands resolved in sodium dodecyl sulfate gels after electrophoresis under denaturating conditions were approx. 1:1:2:2 in the intact complexes and 1:0:0.4:1 in CP2-b. The results were discussed in terms of the functional molecular organization of the Photosystem II reaction center complexes.

Introduction

The primary photochemical event of the PS II reaction center is the charge separation between the donor chlorophyll P-680 and the acceptor pheophytin. The separated charges are stabilized by the secondary electron transfers: oxidized P-680 is reduced by the electron donor Z, while reduced pheophytin gives its electrons to the acceptors Q_A and Q_B (for reviews, see Refs. 1 and 2). The functional components involved in the charge separation and stabilization are associated with the PS II reaction center complex, a specific chlorophyll-protein complex, which consists of five subunits of 47–51, 40–43, 30–34, 27–30 and 9–10 kDa [3–7]. The two large polypeptides of 47–51 and 40–43 kDa carry antenna chlorophyll *a* [5–7], whereas one of the two polypeptides in the 30 kDa region and the 9–10 kDa polypeptide are considered to be the herbicide-binding protein and the apoprotein of cytochrome *b*-559, respectively [4,5].

Recently, we have resolved the PS II reaction center complexes isolated from the thermophilic cyanobacterium *Synechococcus* sp. into two complemental chlorophyll-protein complexes by a mild SDS-polyacrylamide gel electrophoresis [9]. A chlorophyll-protein complex, which we call CP2-b,

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DPC, diphenylcarbazide; PS, Photosystem; CP2-b, Photosystem II reaction center complex lacking a chlorophyll-binding 40 kDa polypeptide; CP2-c, a Photosystem II chlorophyll protein containing only a 40 kDa polypeptide; Chl, chlorophyll.

contains 47, 31, 28 and 9 kDa polypeptides, but no 40 kDa chlorophyll-binding polypeptide. CP2-b shows a high activity of DCIP photoreduction with DPC as electron donor, which is sensitive to heat and SDS. Another chlorophyll-protein complex, CP2-c, contains only the 40 kDa polypeptide, and has a low activity of DCIP photoreduction which is not related to the PS II photochemistry. We concluded therefore that CP2-b represents the functional core of the PS II reaction center complex, and that the 40 kDa polypeptide is not required for the PS II electron transport.

In the present work, photochemical and chemical properties of CP2-b were further characterized in comparison with those of the intact PS II reaction center complexes. Photoreduction of pheophytin and Q_A were measured, and the cytochrome *b*-559 and Mn contents were determined. The relative abundance of the subunit polypeptides of the two complexes was compared.

Materials and Methods

Synechococcus sp. was grown at 55°C for 2–3 days in the light [10,11]. The PS II reaction center complexes were extracted from the thylakoid membranes with 0.8% β-octylglucoside, then purified by digitonin-polyacrylamide gel electrophoresis as described previously [9]. To prepare the PS II reaction center complex lacking the 40 kDa polypeptide (CP2-b) and a chlorophyll-protein containing only 40 kDa polypeptide (CP2-c), the thylakoid membranes were extracted with 0.3% lauryldimethylamine *N*-oxide and, after centrifugation at 230000 × *g* for 40 min, the supernatant was applied to polyacrylamide gels with 0.05% SDS present in the reservoir buffer, but not in gels [9]. Chlorophyll-protein complexes were extracted from homogenized gels and suspended in 50 mM Tris-HCl (pH 7.5) for spectrophotometric assays.

Photoreductions of pheophytin and Q_A were determined with a Hitachi 356 spectrophotometer. Pheophytin photoreduction was measured in the presence of 2–5 mM dithionite and 10–20 μM methyl viologen [12]. Absorption changes in the red region were determined with blue actinic light (350–600 nm, 400 W/m^2). To minimize fluorescence artifacts, cuvettes were placed in the second sample chamber of the spectrophotometer and appropriate interference filters were inserted between the cuvettes and the photomultiplier. Absorption changes at shorter wavelength were measured with the samples in the first sample chamber under illumination with red light (640–800 nm, 2300 W/m^2). The photomultiplier was guarded with a Corning 4-96 filter. To measure photoreduction of Q_A, 5 μM ferricyanide was added to the reaction medium. Samples were placed in the first chamber and illuminated with blue light (350–600 nm, 70 W/m^2). The photomultiplier was protected with 1.5 M $CuSO_4$-saturated $NiSO_4$ solution [13].

Cytochrome *b*-559 was determined with a Hitachi 320 spectrophotometer equipped with a head-on photomultiplier (Hamamatsu TV R-375). Ferricyanide-oxidized–minus–ascorbate- or dithionite-reduced difference spectra were determined with a microprocessor attached to the spectrophotometer. Mn was determined with a Shimazu atomic spectrophotometer AA-640-01 with a flameless graphite furnace. Chlorophyll *a* was determined by the method of Mackinney [14].

Polypeptide compositions of chlorophyll-protein complexes were analyzed by SDS-polyacrylamide gel electrophoresis, as described previously [5,8].

Results

Pheophytin photoreduction

Pheophytin is currently considered to serve as an intermediary electron acceptor of the PS II reaction center [15]. Klimov et al. [12,16] have shown that reduced pheophytin accumulates in the light provided that Q_A is reduced prior to illumination and an efficient reductant of P-680 is present during the illumination. On illumination of the PS II reaction center complexes in the presence of dithionite and methyl viologen, there occurred a rapid absorption increase at 450 nm which returned to the dark level after the light was turned off (Fig. 1). The light-minus-dark difference spectrum shows bleachings at 415, 542 and 685 nm and absorption increases at 448, 550 and 675 nm (Fig. 2), in agreement with the difference spectra for the pheophytin photoreduction [12]. The contents of photoreducible pheophytin were estimated on the basis of chlorophyll *a* present in the complexes by assuming that the differential

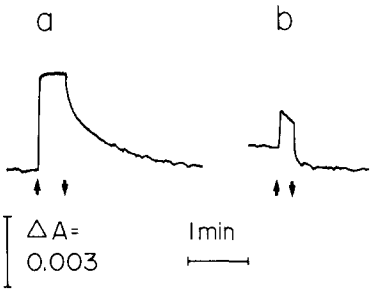

Fig. 1. Light-induced absorbance changes of PS II reaction center complexes (a) and CP2-b (b) at 450 nm. Samples were suspended in 50 mM Tris-HCl (pH 7.5)/2 mM sodium dithionite/10 μM methyl viologen or in 50 μM Tris-HCl (pH 7.5)/5 mM sodium dithionite/20 μM methyl viologen for PS II reaction center complexes or for CP2-b, respectively. Up- and downward arrows indicate times when actinic light was turned on and off, respectively.

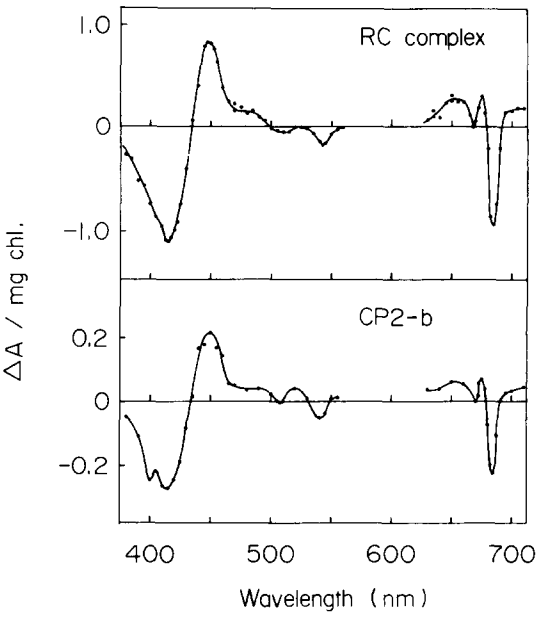

Fig. 2. Light-minus-dark difference spectra of the PS II reaction center complexes and CP2-b.

extinction coefficient of pheophytin reduction at 685 nm as 32 $mM^{-1} \cdot cm^{-1}$ [17] (Table I, the first column). The reaction center complexes have one photoreducible pheophytin per 32 chlorophyll *a*.

Fig. 1b shows light-induced absorption changes in CP2-b under the reducing conditions. The photoresponses were considerably smaller in magnitude and more complex in kinetics as compared with those of the intact reaction center complexes. The initial rapid absorption increase was followed by a slower decrease, and when light was turned off the absorbance decreased rapidly to a level lower than the original level. This suggests that the absorption changes in CP2-b consist of a rapid reversible change and a slow irreversible one. The latter can be ascribed to photobleaching of chlorophyll *a* and/or carotenoids. On the other hand, the difference spectrum for the reversible portion of the absorption changes (Fig. 2) is similar to the spectrum determined with the intact complexes, indicating the occurrence of the pheophytin photoreduction in CP2-b. Because CP2-b has no 40 kDa chlorophyll-binding polypeptide, the results are consistent with our proposal that the 47 kDa protein is the site of the PS II primary photochemistry [6,7,9].

TABLE I

CONTENTS OF REDOX COMPONENTS OF THE PS II REACTION CENTER COMPLEXES AND CP2-b

The figures in parentheses indicate the number of experiments. (A) Total chlorophyll *a* present in the reaction center complexes. (B) Chlorophyll *a* associated with the 47 kDa polypeptide, which was estimated by assuming that chlorophyll *a* distributes between the 47 and 40 kDa polypeptides proportionally to their molecular masses.

Redox components	Reaction center complexes (mol Chl *a*/mol)		CP2-b (mol Chl *a*/mol)
	A	B	
Pheophytin	32± 5 (5)	17±1	137±32 (3)
Q_A	46±13 (4)	25±7	57± 5 (3)
Cytochrome *b*-559	33± 2 (4)	18±2	216±14 (3)
Mn	305±15 (2)	164±8	315±15 (2)

The determination of the photoreducible pheophytin in CP2-b was strongly hampered by the overlapping photobleaching of the antenna pigments. Note that the signal decayed rapidly in CP2-b, suggesting that pheophytin is more easily accessible to oxidants (methyl viologen or oxygen) in the outer environment. Because of its rapid decay as well as its reduced antenna size, CP2-b required an intense light to accumulate reduced pheophytin. The irreversible photobleaching became so rapid at such a high light intensity that the accurate measurement of the reversible changes was difficult. The pheophytin content of CP2-b presented in Table I should therefore be regarded as an underestimate.

Photoreduction of Q_A

Q_A is a bound plastoquinone molecule which serves as one electron carrier between pheophytin and Q_B [1,2]. Because rapid electron transfer from reduced pheophytin to Q_A prevents the charges separated between P-680 and pheophytin from the wasteful recombination, it is of special interest to examine whether or not the photoreduction of Q_A takes place in CP2-b.

Stiehl and Witt [18] have shown that photoreduction of Q_A (X-320) can be monitored by measuring absorption changes in the ultraviolet region. Fig. 3 shows kinetics of light-induced absorption changes at 320 and 260 nm in the two complexes. Illumination of the intact reaction center complexes induced an absorption increase at 320 nm which decayed rapidly and then slowly in the dark (trace a). Absorption changes at 260 nm showed similar kinetics but the sign of the changes was opposite to those at 320 nm. The difference spectrum shown in Fig. 4 is similar to the reported spectra of the Q_A photoreduction [18,19]. The chlorophyll *a*-to-Q_A ratio of 46 which was estimated by assuming the differential extinction coefficient as as 12 $mM^{-1} \cdot cm^{-1}$ at 320 nm [20] is somewhat larger than the chlorophyll-to-pheophytin ratio. This strongly suggests that Q_A is partially solubilized from the complexes during the preparations.

CP2-b also showed light-induced absorption increase at 320 nm and a decrease at 260 nm which relaxed rapidly when light was turned off (Fig. 3b). The difference spectrum with a peak at 320

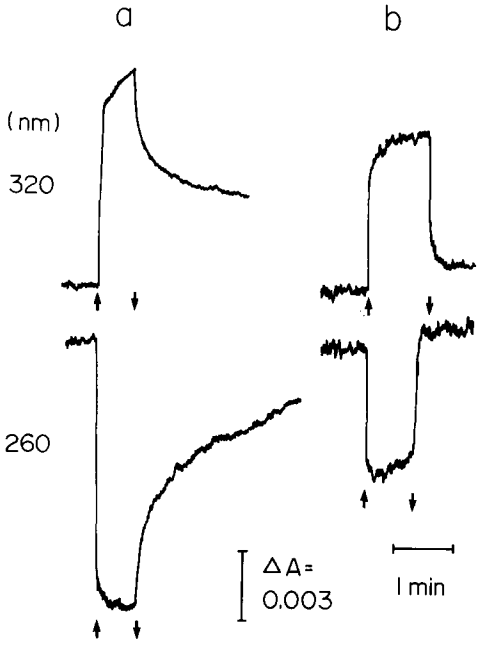

Fig. 3. Light-induced absorbance changes of the PS II reaction center complexes (a) and CP2-b (b) in the ultraviolet region. The reaction mixture contained 50 mM Tris-HCl (pH 7.5) and 5 μM ferricyanide.

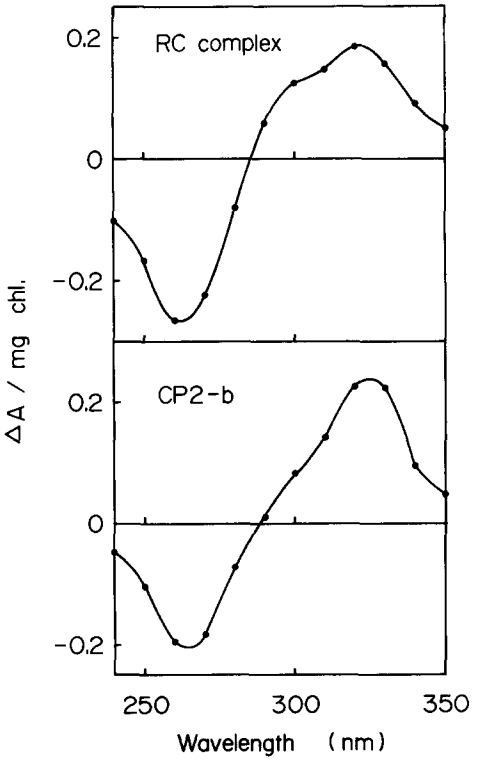

Fig. 4. Light-minus-dark difference spectra of the PS II reaction center complexes and CP2-b in the ultraviolet region.

nm and a trough at 260 nm (Fig. 4) clearly indicates the occurrence of the photoreduction of Q_A in CP2-b. Thus, at least a part of the secondary electron transport proceeds in the complexes. A shoulder at 300 nm was, however, less pronounced in CP2-b, presumably reflecting different contribution of overlapping absorption change(s) between the intact complexes and CP2-b [19]. The rapid dark decay of the photoresponse also suggests that Q_A is more accessible to ferricyanide in CP2-b. The Q_A content of CP2-b was slightly lower than that of the intact complexes on the basis of total chlorophyll *a* (compare the first and third columns of Table I), and hence considerably larger than the pheophytin content of CP2-b. Because of the above-stated difficulty in determining the pheophytin photoreduction in CP2-b, we consider that the Q_A photoreduction is a more reliable measure of the PS II reaction center than the pheophytin photoreduction in CP2-b.

Cytochrome b-559 and Mn contents

The chemically oxidized-minus-reduced difference spectrum was measured to examine cytochromes present in the reaction center complexes and CP2-b (not shown). The two complexes contained cytochrome *b*-559, but no other cytochromes. The chlorophyll *a*-to-cytochrome *b*-559 ratio was 33 for the intact complexes and 216 for CP2-b. About two thirds of the cytochrome in the intact complexes are ascorbate-reducible, whereas the reductant failed to reduce the cytochrome to any appreciable extent in CP2-b.

The two complexes contained only trace amounts of Mn.

Relative abundance of polypeptides

We noticed previously that CP2-b is partially deprived of small subunits as compared with the intact reaction center complexes [9]. In order to examine the extent of the polypeptide deficiency in CP2-b, the two complexes were applied to SDS-polyacrylamide gel electrophoresis under the denaturating conditions, then the relative peak areas of the polypeptide bands resolved and stained with Coomassie blue were determined. The amounts of the two complexes applied to gels were in the ranges where the peak areas are proportional to the amounts of the polypeptides. The peak areas corrected for the molecular weights of respective polypeptides are summarized in Table II. The two polypeptides in the 30 kDa region were not resolved, but the shape of the fused band suggests that the peak areas of the two polypeptides are comparable to each other in the two complexes (cf. Fig. 2, Ref. 9). Thus the relative peak-area ratio of 47/40/31/28/9 kDa polypeptides is approx. 1 : 1 : 1 : 2 for the intact complexes and 1 : 0 : 0.2 : 0.2 : 1 for CP2-b. The two polypeptides in the 30 kDa region decreased in parallel and more markedly than the 9 kDa polypeptide during the preparation of CP2-b.

TABLE II

ABUNDANCE OF POLYPEPTIDES IN THE PS II REACTION CENTER COMPLEXES AND CP2-b

Reaction center complexes and CP2-b were treated with 2.5% SDS/8 M urea/5% 2-mercaptoethanol for 30 min at 25°C, and electrophoresed on 12.5% gels or 10–15% gradient gels. Gels were stained with Coomassie brilliant blue R250. Peak areas of polypeptide bands were determined by scanning with a Shimazu dual-wavelength chromatoscanner CS910 at 560 nm with a reference wavelength at 750 nm, and corrected for molecular weights of respective polypeptides.

Polypeptides (kDa)	Peak area/molecular weight	
	Reaction center complexes	CP2-b
47	1.00	1.00
40	1.00	0.00
28–31	1.78	0.43
9	1.89	1.19

Discussion

PS II reaction center complexes

The PS II reaction center complexes isolated from *Synechococcus* contain each one of pheophytin, Q_A and cytochrome *b*-559 per 32–46 chlorophyll *a*. This is in line with the previous observations that Q_A and cytochrome *b*-559 are present in the PS II reaction center complexes purified from spinach at a ratio of 1 to 40–70 chlorophyll *a* [5], and that the oxygen-evolving preparations isolated from the thermophilic cyanobacterium *Phormidium laminosum* have one pheophytin and one Q_A per 44 chlorophyll *a* [21].

The relative abundance of subunit polypeptides in the reaction center complexes was also ex-

amined in the present work. If all the polypeptides of the PS II reaction center complexes have the same affinity for Coomassie blue, the peak area of polypeptides resolved by SDS gel electrophoresis implies a simple stoichiometry of one 47, one 40, one 31, one 28 and two 9 kDa polypeptides. This, together with 32 chlorophyll *a* molecules, gives tentatively a minimum molecular mass of the complex of about 200 000.

CP2-b

We showed previously [6,7] that chlorophyll proteins containing the 47 kDa polypeptides emit a fluorescence band at 697 nm at the liquid nitrogen temperature. More recently, CP2-b was found to show high rates of DCIP photoreduction which is sensitive to SDS and heat [9]. Because the PS II reaction center complexes have two, and only two, chlorophyll-binding polypeptides, and CP2-b contains only the 47 kDa chlorophyll-binding polypeptide [6,8], we concluded that the 47 kDa polypeptide is the site of the primary photochemistry of PS II [9]. The finding that the photoreduction of pheophytin takes place in CP2-b is consistent with our conclusion.

The occurrence of pheophytin photoreduction in the 47 kDa polypeptide has previously been suggested by Nakatani with PS II preparations from spinach [22]. However, the difference spectrum of spinach preparations was obscured by overlapping large irreversible absorption changes, and a chlorophyll protein containing 43 kDa polypeptide (corresponds to CP2-c in *Synechococcus*) showed a light-induced reversible absorption change comparable in size to that of the 47 kDa chlorophyll-protein. In contrast, our difference spectrum bears all spectral characteristics of the pheophytin photoreduction, and CP2-c did not exhibit a reversible photoresponse (data not presented). Thus the PS II preparations from the thermophilic cyanobacterium provide more convincing evidence for the localization of the primary photochemistry on the 47 kDa polypeptide.

Especially noteworthy is the finding that photoreduction of the secondary electron acceptor Q_A occurs in CP2-b. This indicates that CP2-b can carry out not only the charge separation, but also the stabilization of the separated charges. The finding therefore provides a strong support for our view that CP2-b represents the functional core of the PS II reaction center complexes. The occurrence of at least a part of the secondary electron transport in CP2-b has been predicted from the high activity of DCIP photoreduction in this complex [9].

As stated above, the Q_A photoreduction is a better measure of the PS II photochemistry than the pheophytin photoreduction in CP2-b. CP2-b has one photoreducible Q_A per 57 chlorophyll *a*, which corresponds to 48% of the Q_A content of the intact reaction center complexes on the basis of chlorophyll *a* associated with the 47 kDa polypeptide. The activity of DCIP photoreduction in CP2-b can be related to its Q_A content: the rate of DCIP photoreduction in CP2-b is comparable to that in the reaction center complexes on the basis of total chlorophyll *a* [9], and hence is roughly a half that of the reaction center on the basis of chlorophyll *a* associated with the 47 kDa polypeptide. In contrast, the quantum yield of DCIP photoreduction in CP2-b is only less than 20% of that in the intact reaction center complexes [9]. The quantum yield of CP2-b must have also been lowered by inactivation of other secondary electron transport, accelerated back reaction, or modification of the antenna pigment system.

The present work revealed interesting features of the association of subunit polypeptides and functional components in the PS II reaction center complexes. The SDS gel electrophoresis, which completely liberated the 40 kDa polypeptides from the complexes, caused 76% decrease in the 28 and 31 kDa polypeptides and 38% decrease in the 9 kDa polypeptide. This indicates that the association with the 47 kDa polypeptide is resistant to the detergent in the order of the 9, 28 and 31, and 40 kDa polypeptides. Interestingly, the 28 and 31 kDa polypeptides always decreased in parallel, suggesting a close structural relationship between the two polypeptides.

The chlorophyll-protein binding is considerably stronger than the protein-protein association between 47 and 40 kDa subunits, or between the 47 kDa and the two subunits in the 30 kDa region. In contrast, the cytochrome *b*-559 content of CP2-b, which is less than 10% of that in the intact complexes, indicates that SDS preferentially solubilized the heme moiety of the cytochrome, leaving

the 9 kDa apoprotein attached to the 47 kDA polypeptide. Note that the amount of Q_A remaining in CP2-b (48%) is significantly larger than those of the two polypeptides in the 30 kDa region (24%). This tends to suggest that Q_A is located on the 47 kDa polypeptide rather than on one of the two 30 kDa polypeptides. Finally, the intact reaction center complexes and CP2-b contain only very small amounts of Mn. This explains why the reaction center complexes and CP2-b cannot use water as electron donor.

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