Simple and discrete isolation of an O_2-evolving PS II reaction center complex retaining Mn and the extrinsic 33 kDa protein

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An O_2-evolving PS II reaction center complex retaining Mn and the extrinsic 33 kDa protein with almost the same relative abundance as in situ (denoted as RCII[33,Mn] complex) was prepared by a simple one-step sucrose density gradient centrifugation of octylglucopyranoside (OGP)-solubilized PS II membranes The complex evolved O_2 at a high rate of 550–850 μmol/mg Chl per h with ferricyanide as electron acceptor in the presence of $CaCl_2$ and digitonin The protein composition analysis of this complex revealed that OGP treatment discretely dissects the PS II membranes into RCII[33,Mn] complex and LHCP, and the resulting RCII[33,Mn] complex preserves the distinct structural integrity for O_2 evolution in situ

Oxygen evolution *PS II* *Reaction center* *33 kDa protein* *Octylglucopyranoside* *Photosynthesis*

1. INTRODUCTION

Yuasa et al. [1] isolated the spinach PS II reaction center complex retaining Mn and the extrinsic 33 kDa protein by solubilizing Triton PS II membranes [2] with OGP, and provided a working hypothesis that such a complex is a structural minimum for O_2 evolution. However, the complex they obtained then did not show O_2 evolution.

The hypothesis was recently proved by successful preparation of such complexes capable of O_2 evolution Tang and Satoh [3] isolated an O_2-evolving reaction center complex by two-step column chromatographic separation of digitonin-solubilized chloroplasts. Satoh et al. [4] also isolated a similar O_2-evolving reaction center complex from a thermophilic cyanobacterium by combination of density gradient centrifugation followed by two-step column chromatographic separation of OGP-solubilized thylakoids

Here we report a simple procedure to prepare the O_2-evolving reaction center complex by one-step sucrose density gradient of OGP-solubilized PS II membranes. The photochemical properties and protein composition analysis of this complex have shown that OGP effects a discrete dissection of the PS II membranes into the unit for O_2 evolution and LHCP.

2. MATERIALS AND METHODS

O_2-evolving PS II membranes were prepared from spinach according to Berthold et al. [2] with slight modifications described in [5]. Membranes were washed with an MSN medium containing

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Abbreviations PS II, photosystem II, Mes, 4-morpholineethanesulfonic acid, DCIP, 2,6-dichlorophenolindophenol, DPC, 1,5-diphenylcarbazide, SDS-PAGE, SDS-polyacrylamide gel electrophoresis, LDS, lithium dodecyl sulfate, OGP, 1-*O*-*n*-octyl-β-D-glucopyranoside, Chl, chlorophyll, LHCP, light-harvesting chlorophyll *a*/*b*-protein complex, DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

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0.4 M sucrose, 10 mM NaCl and 40 mM Mes-NaOH (pH 6.5) and solubilized with 60 mM OGP, 10 mM NaCl and 20 mM Mes-NaOH (pH 6.5) at a Chl concentration of about 1 mg/ml. Immediately after mixing, insoluble gray materials were removed by centrifugation (20500 × *g*, 10 min), and the resulting green supernatant (1 ml) was layered onto a 10–30% linear gradient (5 ml) of sucrose solution containing 60 mM OGP, 10 mM NaCl, 20 mM Mes-NaOH (pH 6 5), and then centrifuged with a Hitachi RP83T angle rotor (224000 × *g*, 5 h). The colorless fraction and the Chl-containing bands were collected separately by a syringe, and the remaining pellet was suspended in MSN medium.

One dimensional SDS-urea-PAGE was done according to [6] provided that 5.5 M urea was included in the stacking and separating gels. We usually used a composite separating gel containing 12.5 and 14.5% acrylamide in the upper two-thirds and lower one-third, respectively, to give a better resolution. Apparent molecular masses of the separated proteins were determined with a uniform separating gel of 14 5% acrylamide. Samples were solubilized in 2.5% SDS, 125 mM dithiothreitol, 10 mM NaCl, 40 mM Mes-NaOH (pH 6 5) and sucrose (≧5% sucrose), and electrophoresed at room temperature with a constant current less than 10 mA. Gels were stained with Coomassie brilliant blue R250 (Biorad) and scanned at 560 nm relative to 750 nm with a Shimadzu dual-wavelength chromatoscanner (CS-900) to determine the relative abundance of the proteins.

Two-dimensional gel electrophoresis was done with a combination of LDS-PAGE in the 1st dimension and SDS-urea-PAGE in the 2nd dimension The 1st LDS-PAGE was performed at 4°C in the dark according to [7] provided that LDS was used instead of SDS Samples were solubilized with LDS (LDS/Chl = 1 5), 30 mM OGP and 50 mM dithiothreitol, and electrophoresed in a separating gel containing 10% acrylamide After electrophoresis, each lane of the 1st slab gel was cut into a strip with a razor blade. The gel strip was equilibrated with 2% SDS and fixed onto the above-mentioned composite slab gel with agarose according to [8], and subjected to SDS-urea-PAGE for the 2nd dimension.

O_2 evolution was measured with a Clark-type O_2 electrode at 25°C in 0.4 M sucrose, 1 mM ferricyanide, 10 mM NaCl and 40 mM Mes-NaOH (pH 6.5). DCIP photoreduction was measured spectrophotometrically at 600 nm in 0.4 M sucrose, 10 mM NaCl, 60 μM DCIP, and 40 mM Mes-NaOH (pH 6.0). Saturating actinic light was provided by a xenon lamp passing through a $CuSO_4$ solution, a red filter (Toshiba, VR65) and a heat-absorbing filter (Nihon Shinku, cold filter B). Mn abundance was determined with a Shimadzu atomic absorption spectrophotometer (AA-640-13) equipped with a graphite furnace atomizer (GFA-3) as described in [5]. Chl concentration and Chl *a*/*b* ratio were determined according to Arnon [9].

3. RESULTS

Two green bands were separated on the sucrose density gradient as illustrated in table 1. The colorless top fraction contained little Chl. About 80% of Chl and the rest were recovered in the upper green band and the lower green band, respectively Two to 3% of Chl were found in the green pellet. Of these fractions, the upper green band had a low Chl *a*/*b* ratio, indicative of enrichment in LHCP, while the lower green band and the green pellet had higher Chl *a*/*b* ratios, indicative of enrichment in PS II reaction center complex. As will be discussed later, the complex in the green pellet preserves the extrinsic 33 kDa protein and Mn.

Although the green pellet preserved an appreciable activity of DCIP photoreduction with DPC as donor, both activities of DCIP photoreduction with water as donor and O_2 evolution were lost to a great extent during OGP treatment. However, when $CaCl_2$ was included in the reaction mixture for activity measurement particularly in the presence of digitonin, both activities were surprisingly enhanced by a factor of 2–10 by $CaCl_2$ (5 mM) and by an additional factor of about 1.5 by digitonin (0.1%), and the O_2 evolution by the green pellet reached as high as 590 μmol O_2/mg Chl per h at the optimum pH of 6.5 with 1 mM ferricyanide as electron acceptor (table 1). The activity enhancement was specific for $CaCl_2$ and digitonin; other salts, NaCl, $MgCl_2$, $Ca(NO_3)_2$ and $MnCl_2$ were much less effective as compared with $CaCl_2$ (table 2), and other detergents (OGP and Triton X-100 were tested) were not effective at all (not shown). DCIP photoreduction with DPC

Table 1

Photochemical activities and Mn abundance of the fractions of sucrose density gradient centrifugation

Fraction	Chl *a/b*	$H_2O \rightarrow DCIP$	$DPC \rightarrow DCIP$	O_2 evolution (μmol O_2/mg Chl per h)	Mn (atom/200 Chl)
		(μmol DCIP/mg Chl per h)			
PS II membranes	2 0	425	557	445[a]	3 6
colorless band	(2 0)	–	–	–	–
upper green band	1 5	0	8	–	0 3
lower green band	14 1	0	55	0[b]	4 5
green pellet	19 7	18	253	44[b]	13 2
		(69)[c]	(232)[c]	(592)[c]	

[a] Dichlorobenzoquinone (0.3 mM) was used as electron acceptor
[b] Ferricyanide (1 mM) was used as electron acceptor
[c] Values in parentheses were obtained in the presence of digitonin (0 1%) and $CaCl_2$ (5 mM)

as donor was enhanced neither by $CaCl_2$ nor by digitonin. The high rate of O_2 evolution by the green pellet fraction is consistent with the high content of Mn in this fraction (table 1) Assuming 60 Chl/P680 in this type of complex according to Satoh and Mathis [10], the Mn abundance in this fraction is calculated to be 4.0 Mn atoms per reaction center. We denote here the complex in this fraction as RCII[33,Mn].

Another notable characteristic of the photochemical activity of RCII[33,Mn] is its modified affinity to electron acceptors. As demonstrated in table 3, ferricyanide and dichlorobenzoquinone were the only efficient electron acceptors for the O_2 evolution by this complex, while the PS II membranes could utilize various acceptors at comparable efficiencies. In

Table 2

Effects of salts on O_2 evolution by RCII[33,Mn] (the green pellet) measured in the basal reaction medium containing 0 4 M sucrose, 1 mM ferricyanide, 0 1% digitonin and 40 mM Mes-NaOH (pH 6 5)

Addition	O_2 evolution (μmol O_2/mg Chl per h)
None	21
10 mM NaCl	286
10 mM $NaNO_3$	140
5 mM $Ca(NO_3)_2$	498
5 mM $Ca(NO_3)_2$ + 10 mM NaCl	835
5 mM $CaCl_2$	809
None	10
5 mM $CaCl_2$	546
5 mM $MgCl_2$	120
5 mM $MnCl_2$	80

Table 3

Acceptor dependency of O_2 evolution by RCII[33,Mn] (green pellet fraction) and PS II membranes measured polarographically for ferricyanide and quinones and expressed as μmol O_2/mg Chl per h, and spectrophotometrically for DCIP and expressed as μmol DCIP/mg Chl per h

Acceptors	RCII[33,Mn]		PS II membranes	
	None	+DG	None	+DG
Ferricyanide (1 mM)	536	846	80	43
Dichlorobenzoquinone (0 3 mM)	273	461	604	582
Dimethylbenzoquinone (1 mM)	0	2	391	348
Duroquinone (0.3 mM)	0	0	251	256
Phenylbenzoquinone (0 3 mM)	3	2	608	622
DCIP (0 06 mM)	29	55	306	338

$CaCl_2$ (5 mM) is included in the reaction medium instead of NaCl Digitonin (0.1%) is also included if stated as +DG

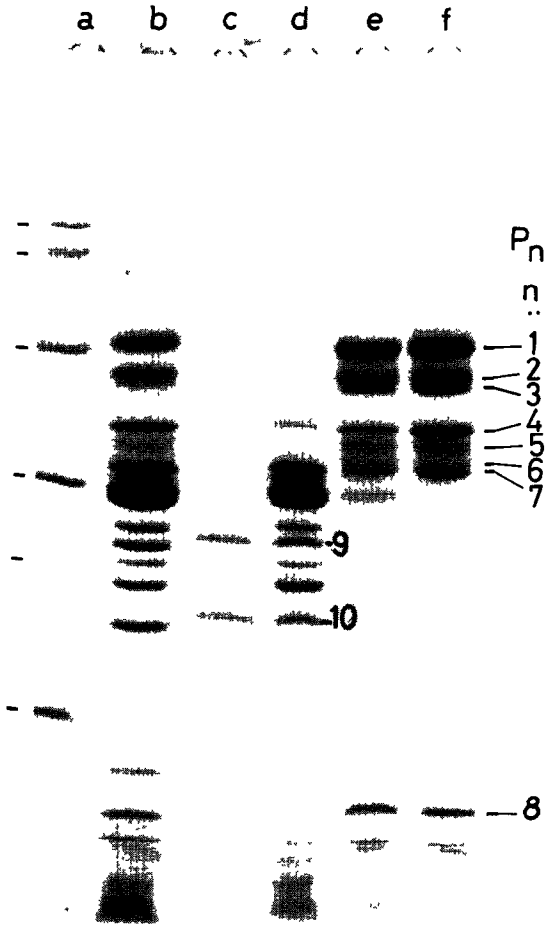


Fig.1. Protein composition analyzed by SDS-urea-PAGE (a) Molecular marker, (b) PS II membranes, (c) colorless fraction, (d) upper green fraction (LHCP), (e) lower green fraction, (f) green pellet fraction (RCII[33,Mn]). Proteins found in RCII[33,Mn] complex are numbered as P_n (n = 1–8) and the two extrinsic proteins (23–24, 16–18 kDa) as P_9 and P_{10}, respectively.

addition, the O_2 evolution by this complex with ferricyanide as acceptor was insensitive to 5 μM DCMU (not shown) These properties indicate that the RCII[33,Mn] complex retains native O_2-evolving center although the complex has been damaged on the acceptor side, probably on Q_B, the secondary quinone electron acceptor of PS II.

The protein compositions of the 4 fractions are shown in fig.1. The green pellet (RCII[33,Mn]) had a simple composition consisting of 8 proteins (denoted P_1–P_8), whose apparent molecular masses determined on a uniform gel (14.5% acrylamide) were 46.3, 41.2, 40.4, 34.5, 35.6, 32.7, 31.9 and 9.9 kDa, respectively (the discrepancy between P_4 and P_5 with respect to the molecular mass and the migration distance in fig.1 (lane f) is due to their unusual mobilities in gels with different acrylamide concentrations). Most of these proteins were assigned by referring to the results reported by Satoh et al. [3,11,12]: P_1, the 47 kDa P680 containing Chl protein; P_2 and P_3, the 43 kDa Chl containing protein and its proteolysis product (see section 4); P_4, the extrinsic 33 kDa protein; P_5, P_6 and P_7, the intrinsic 34 kDa protein and the 32 kDa herbicide-binding protein by Satoh et al. [12], and one more unknown protein; P_8, cytochrome *b*-559.

Table 4 shows the relative abundance of these proteins estimated on the basis of 47 kDa reaction center pigment protein (P_1). All the proteins found in the RCII[33,Mn] complex (excepting P_6 and P_7) showed relative values very similar to those of corresponding proteins in the initial PS II membranes. These results indicate that the RCII[33,Mn] complex recovered in the green pellet preserves the structural integrity of the O_2-evolving

Table 4

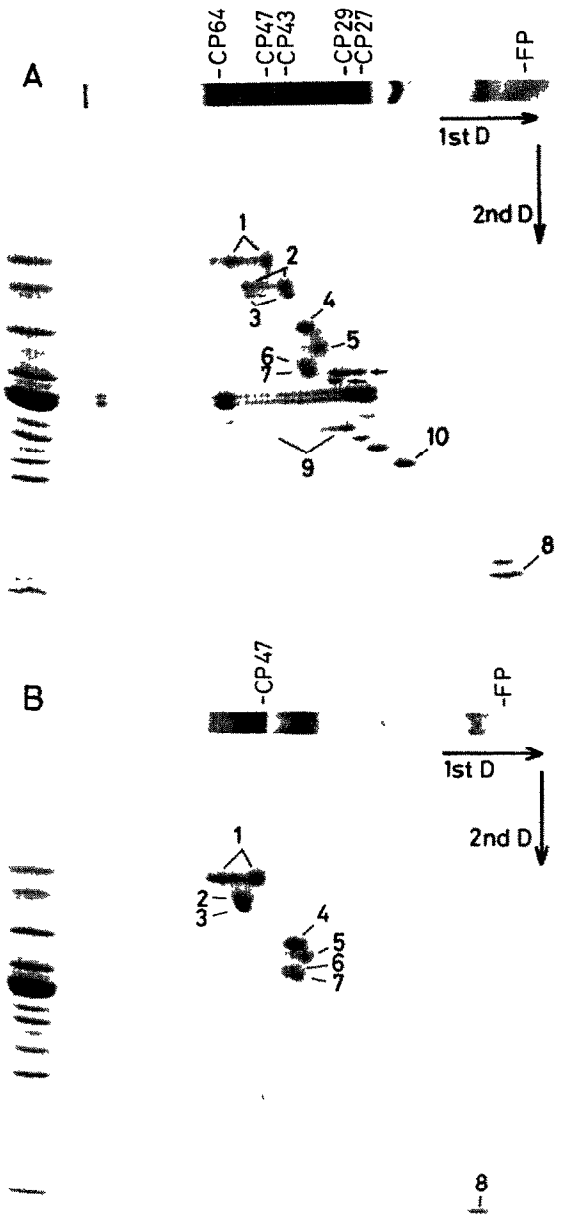
Relative abundance (%) of the proteins found in the green pellet fraction (RCII[33,Mn]) and the lower green fraction

Protein	PS II membranes	Green pellet fraction (RCII[33,Mn])	Lower green fraction
P_1 (47 kDa)	100	100	100
$P_2 + P_3$ (43 kDa)	75	71	74
P_4 (extrinsic 33 kDa)	65	57	33
P_5[a]	31	24	32
$P_6 + P_7$[a]	–	37	41
P_8 (cytochrome *b*-559)	31	19	32

[a] See section 4 as to the assignment of these proteins

center in situ, and also that OGP effects a discrete separation between this complex and LHCP

Fig.1 also shows that LHCP-originating proteins (26–30 kDa) were exclusively found in the upper green band, although this fraction contained several other proteins including the extrinsic 33 kDa protein. Of these, the two proteins, P_9 and P_{10}, were assigned to the extrinsic 23–24 and 16–18 kDa proteins in spinach PS II membranes, respectively, based on their behavior on extraction with 1 M NaCl (not shown). The protein composition of the lower green band was almost the same as that of RCII[33,Mn] with the exception of a lower relative content of the extrinsic 33 kDa protein and slight contamination by some of the LHCP-originating proteins. The low content of 33 kDa protein is consistent with the low Mn abundance and low O_2 evolution by this fraction (tables 1 and 2). Taking these into account, a notable feature of the present separation method may be pointed out as follows: the protein compositions of the green pellet (RCII[33,Mn]) and the upper green band (LHCP) are in a discrete complementary relation to each other with an exceptional ambiguity only around the 32 kDa region, namely, whether or not P_6 and P_7 are inherent in the RCII[33,Mn] complex



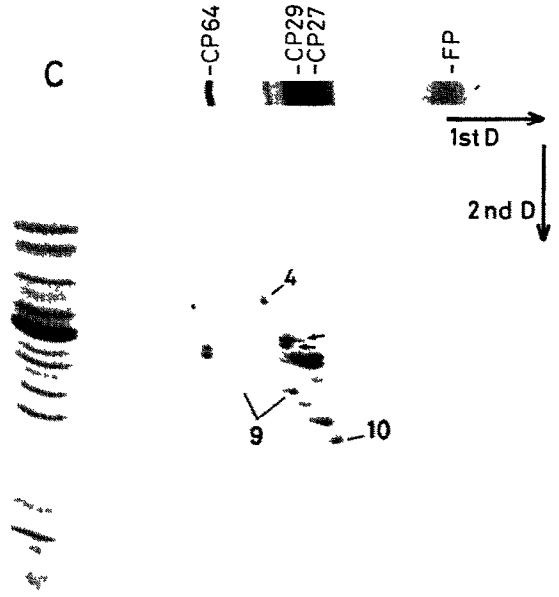


Fig 2 Two-dimensional electrophoresis of PS II membranes (A), green pellet fraction (RCII[33,Mn]) (B), and upper green fraction (LHCP) (C) Numbers of the protein spots are those of P_n defined in fig 1 The profiles on the top are the LDS-PAGE profiles in the 1st dimension for the respective two-dimensional gels Chl-containing bands are denoted according to [7] The vertical profiles in the left part are those of the PS II membranes obtained by simultaneous electrophoresis in the 2nd dimension Arrows stand for the two proteins contained in the strong 32 kDa bands in fig 1 lane d (see text)

Since the ambiguity arises from the difficulty in distinguishing P_6 and P_7 bands in the RCII[33,Mn] fraction from the strong band around the 32 kDa region in the LHCP fraction, we tried to separate them by means of two-dimensional electrophoresis Although P_6 and P_7 were electrophoresed in close proximity in both dimensions, it was clearly shown that the two spots of P_6 and P_7 identified in the RCII[33,Mn] fraction (fig.2B) were found in the original PS II membranes (fig.2A) but not at all in the LCHP fraction (fig.2C). The two-dimensional profile for PS II membranes (fig.2A) demonstrates the presence of another pair of spots (with arrows) near the two spots of P_6 and P_7. These were identified with the corresponding spots (with arrows) in the LHCP fraction (fig.2C); namely, the strong 32 kDa band in LHCP (fig.1) is a composite band of these two proteins (with arrows). The migration distances of these spots were appreciably different from those of P_6 and P_7 in LDS gel (1st dimension), even though being almost the same in SDS-urea gel (2nd dimension). This enabled successful distinction between the P_6 and P_7 band(s) in the RCII[33,Mn] fraction and the two proteins for the 32 kDa bands in the LHCP fraction. It was thus clearly indicated that P_6 and P_7 are specifically associated with the RCII[33,Mn] complex as inherent components

4. DISCUSSION

The RCII[33,Mn] complex we obtained here preserves the extrinsic 33 kDa protein and Mn at a relative abundance almost the same as in situ. These characteristics are very similar to those of the crude OGP-complex prepared by Yuasa et al. [1]. Since the latter complex contained a small amount of LHCP and some other proteins, they could not confirm whether its residual O_2 evolution was inherent in this complex Our RCII[33,Mn] complex, however, contained no LHCP, so that we can conclude that the observed activity is inherent in this complex

The RCII[33,Mn] complex evolved O_2 at a rate of 550–850 μmol/mg Chl per h under the best conditions. This rate was comparable to or higher than those (150 and 300–400 μmol O_2/mg Chl per h, respectively) reported for similar complexes from spinach by Tang and Satoh [3] and from a cyanobacterium by Satoh et al. [4]. The O_2 evolution by these complexes was commonly enhanced by $CaCl_2$, probably because of the absence of extrinsic proteins (23–24 and 16–18 kDa) in these complexes [13,14]. Satoh and Katoh [15] reported a crucial requirement of digitonin for O_2 evolution by their cyanobacterial crude complex, and similarly, digitonin enhanced O_2 evolution by our RCII[33,Mn] complex (table 3). No activity enhancement has, however, been reported for the spinach complex by Tang and Satoh [3] or for the purified cyanobacterial complex by Satoh et al. [4]. This is probably because these complexes would have contained some amount of digitonin, since the final steps of purification were done in the presence of digitonin. Taking these into account, we consider that the O_2 evolution by these types of complexes would commonly show the enhancement by digitonin.

The OGP treatment seems to cause considerable damage on the acceptor side, as shown by the loss of DCMU sensitivity or by the marked change in the acceptor dependency spectrum between the RCII[33,Mn] complex and the initial PS II membranes (table 3). Although a detailed comparison of the spectrum between our RCII[33,Mn] complex and those by Tang and Satoh [3] or by Satoh et al. [4] is difficult because only a few acceptors were tested with their complexes, it seems likely that the spectrum differs greatly from the others. Our RCII[33,Mn] complex efficiently transfers electrons to ferricyanide and dichlorobenzoquinone, while that of Tang and Satoh does so efficiently to phenylbenzoquinone and DCIP [3], and that of Satoh et al efficiently to ferricyanide or ferricyanide plus phenylbenzoquinone [4] but poorly to dichlorobenzoquinone [15]. Probably, OGP and digitonin give rise to different extents of damage in spinach membranes, and OGP effects various degrees of damage between spinach and cyanobacterial thylakoids.

The proteins found in our RCII[33,Mn] complex are assigned as follows: P_1 is the apoprotein of CP47 [7], the so-called 47 kDa P680-carrying protein. Similarly, both P_2 and P_3 are the apoprotein of CP43 [7], the so-called 43 kDa protein. Judging from the fluctuation in P_2/P_3 ratio dependent on the source of spinach, P_3 would probably be a proteolysis product from P_2 formed during preparation of chloroplasts and/or PS II membranes. P_4 is assigned to the extrinsic 33 kDa protein based on

its behavior on extraction with 1 M $CaCl_2$ and 0.8 M Tris One of the 3 proteins in the 32 kDa region (P_5–P_7) would be the so-called herbicide-binding protein based on the report by Satoh et al. [12]. P_8 is probably cytochrome *b*-559 based on its molecular mass. Other extrinsic proteins, P_9 and P_{10}, which are assigned to 23–24 and 16–18 kDa proteins in spinach PS II membranes, respectively, were not found in the RCII[33,Mn] complex. This protein composition is more or less the same as that of Tang-Satoh's complex [3,12] which consisted of 47 kDa, 43 kDa, extrinsic 33 kDa and 10 kDa proteins and two additional proteins around 30 kDa. Thus, two of the 3 proteins in the 32 kDa region (P_5–P_7) would probably correspond to these two proteins around 30 kDa. Since P_6 and P_7 are closely electrophoresed on various types of gel but are always split into two bands with reproducibly different staining intensities, both would be inherent in the RCII[33,Mn] complex The larger number of proteins around 32 kDa in our complex would have resulted from the higher resolution in the present study.

The lower green fraction had a protein composition similar to the RCII[33,Mn] complex (fig 1), but the abundance of the extrinsic 33 kDa protein and Mn was much less than those of RCII[33,Mn] complex, and did not evolve O_2 at all. These characteristics are similar to those of the purified OGP complex of Yuasa et al. [1]. Judging from the band position on the density gradient, the particle size of the complex in this fraction appears far smaller than those of RCII[33,Mn]. The partial loss of 33 kDa protein and Mn atoms does not account for the big difference in particle size Probably, the RCII[33,Mn] complex in the green pellet fraction exists as an oligomer, while the complex in the lower green fraction exists as a monomer.

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