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Isolation of an oxygen-evolving Photosystem II preparation containing only one tightly bound calcium atom from a chlorophyll *b*-deficient mutant of rice

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Oxygen-evolving Photosystem II (PS II) particles were prepared from the thylakoid membranes of a chlorophyll *b*-less rice mutant, which totally lacks light-harvesting chlorophyll *a*/*b* proteins, after solubilization with β-octylglucoside. The preparation was essentially free of Photosystem I as judged from its low-temperature fluorescence spectrum and polypeptide composition. The PS II particles contained all the major subunit polypeptides of the PS II reaction center core complexes and the three extrinsic proteins related to oxygen evolution. The relative abundances of the 33, 21 and 15 kDa proteins were 100, 64 and 20%, respectively, of the corresponding proteins in the mutant thylakoids. The chlorophyll-to-Q_A ratio was 53 and there was only one bound Ca^{2+} per Q_A. Thus, one of the two bound Ca^{2+} present in the oxygen-evolving PS II membrane preparations from wild-type rice (Shen J.-R., Satoh, K. and Katoh, S. (1988) Biochim. Biophys. Acta 933, 358–364) is missing. The mutant PS II particles were highly active in oxygen evolution in the absence of exogenously added Ca^{2+}, although addition of 5 mM Ca^{2+} enhanced the activity by 30%. When the 21 and 15 kDa proteins were supplemented to the particles, the Ca^{2+}-effect disappeared and the rate of oxygen evolution increased to a level exceeding 1000 μmol O_2 per mg chlorophyll per h. The results indicate that the number of Ca^{2+} needed to promote a high rate of oxygen evolution is one per PS II in higher plants.

Introduction

Calcium is currently considered as an important cofactor in photosynthetic oxygen evolution but the function and number of the metal cation involved in PS II electron transport are still in dispute [Refs. 1–17, also see for reviews Refs. 18–22]. The oxygen-evolving PS II preparations isolated from the thermophilic cyanobacterium *Synechococcus* sp. contain only one bound Ca^{2+} per PS II when contaminating Ca^{2+} is removed by treatment with a cation chelating resin, chelex 100 [13]. Highly purified oxygen-evolving complexes from *Synechococcus* contain 0.46–0.93 Ca^{2+} per PS II and there is a good correlation between the Ca^{2+} content and the rate of oxygen evolution [9]. Thus, only one bound Ca^{2+} is needed for oxygen evolution in the cyanobacterium. Spectrophotometric studies suggest that the Ca^{2+} functions near the PS II reaction center [7].

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LHC II, light-harvesting chlorophyll protein complex of Photosystem II; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; PS, Photosystem; Chl, chlorophyll.

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Oxygen-evolving PS II membrane preparations isolated from higher plants are more abundant in Ca^{2+} than the cyanobacterial preparations. Cammarata and Cheniae [14] have shown that spinach and wheat PS II membranes contain about 2 and 3 Ca^{2+} per 200 chlorophyll when adventitious Ca^{2+} is removed with EGTA and A23187, an ionophore specific to divalent metal cations. We have also determined the Ca^{2+} contents of three oxygen-evolving PS II membrane preparations including a rice preparation, which shows the highest level of oxygen-evolving activity so far reported, by the chelex method [17]. Irrespective of large differences in their activity, all the three preparations contain 2 Ca^{2+} per PS II. We concluded therefore that the maximum number of Ca^{2+} needed to promote a high rate of oxygen evolution is two per PS II in higher plants.

An important question arises as to whether PS II electron transport of higher plant differs from that of the cyanobacterium in respect to the number of functional Ca^{2+}. Ca^{2+} has been postulated to function at or near the site of water oxidation in higher plants [2,5,8,15]. Evidence obtained in earlier works is, however, indirect because the function of Ca^{2+} has been deduced mainly from reactivation of oxygen evolution or related reactions by addition of Ca^{2+} to PS II membrane preparations washed with concentrated NaCl. We have shown recently that the NaCl-wash does not affect the abundance of Ca^{2+} bound to the PS II membranes and proposed that Ca^{2+} restores the functional conformation of the salt-washed preparations which lack the extrinsic 23 kDa protein [17]. Our results are, however, at variance with the observation of Cammarata and Cheniae, who reported that treatment of PS II membranes with concentrated NaCl diminishes the Ca^{2+} content to one Ca^{2+} per PS II [14]. The cause for this discrepancy is not clear at present.

The Ca^{2+} abundance was also nearly halved by treatment of spinach PS II membranes with a citrate solution at pH 3.0 [23]. The loss of Ca^{2+} was accompanied by a strong inactivation of oxygen evolution and the lost activity was restored by addition of Ca^{2+}. We have shown that oxygen-evolving complexes purified from PS II membranes with β-octylglucoside [24] have one Ca^{2+} per PS II reaction center [17]. The purified complexes show a strong Ca^{2+} requirement for oxygen evolution [24]. However, this cannot be attributed solely to the loss of Ca^{2+} because the detergent also solubilized the extrinsic 23 and 17 kDa proteins. In fact, the Ca^{2+} concentration dependency of the purified complexes is similar to that of the NaCl-washed PS II membranes containing two bound Ca^{2+}, suggesting that the Ca^{2+} requirement is related to the release of the 23 kDa extrinsic protein rather than the loss of Ca^{2+} [17]. The Ca^{2+} abundance of spinach PS II membranes was also decreased to about one Ca^{2+} per PS II by treatment with chelex 100 in the presence of digitonin without significant loss of the oxygen-evolving activity [16]. Although the effect of the treatment on the Ca^{2+} content was somewhat variable with preparations, the data suggest that the extracted Ca^{2+} is not needed for PS II electron transport. Thus, all the data show that the two Ca^{2+} have different binding affinities: one Ca^{2+} binds very tightly to a subunit polypeptide of the oxygen-evolving complex, while another Ca^{2+} is readily released by various treatments. However, there are marked controversies as to the function of the extractable Ca^{2+} in PS II electron transport of higher plants.

In the present work, we have isolated an oxygen-evolving PS II preparation from a rice chlorina mutant. Chlorophyll-protein compositions of the mutant have recently been well characterized [25–27]. The mutant belongs to a group of ten mutant strains, which are totally incapable of synthesizing chlorophyll *b* and hence lack light-harvesting chlorophyll *a/b* proteins. Thus, the rice mutant yielded PS II particles, which are completely free from antenna chlorophyll proteins. The preparations contain only one Ca^{2+} per PS II but are still able to evolve oxygen at substantial rates in the absence of exogenously added Ca^{2+}.

Materials and Methods

Wild-type (*Oryza sativa* L. var. Nipponbare) and the chlorophyll *b*-deficient mutant of rice (chlorina 9) were grown for 5–7 weeks in a greenhouse under natural light conditions [28]. Spinach was obtained from a local market. The thylakoid membranes were prepared as described

previously [25], using a medium containing 0.4 M sucrose, 50 mM Hepes/NaOH (pH 7.5), 10 mM NaCl, 0.2% bovine serum albumin and 2 mM $MgCl_2$. The isolated membranes were suspended in 40 mM Mes/NaOH (pH 6.5), 10 mM NaCl and 5 mM $MgCl_2$ (MNM medium) containing 0.4 M sucrose and stored at 77 K. Oxygen-evolving PS II membrane fragments were prepared from spinach and rice thylakoid membranes according to the method of Berthold et al. [29], which was modified as described previously [17]. A PS II preparation was isolated from the thylakoid membranes of the chlorina mutant by a procedure which had been developed for the preparation of oxygen-evolving particles from the thermophilic cyanobacterium, *Synechococcus* sp. [6]. The membranes were treated with 50 mM β-octylglucoside in the MNM medium containing 0.16 M sucrose and 5 mM $CaCl_2$ for 5 min. The chlorophyll concentration was 1 mg/ml. The suspension (0.5 ml) was layered on a 0.5 M sucrose cushion in the MNM medium (5 ml) and centrifuged at 63 000 × *g* for 15 min. A green band appeared at the interface between the cushion and the upper layer contained PS II preparations, while essentially all PS I and some PS II were found in pellets at the bottom of the tubes. The chlorophyll recovered in the PS II preparations accounted for 10–30% of the total chlorophyll presented in the thylakoid membranes. The PS II fraction was collected by centrifugation at 230 000 × *g* for 30 min and suspended in the MNM medium containing 0.4 M sucrose.

Absorption spectra were determined with a Hitachi 320 spectrophotometer at room temperature. Fluorescence emission spectra were determined at 77 K as described previously [25]. Q_A was measured spectrophotometrically at 325 nm in the presence of 10 μM ferricyanide and 10 μM DCMU [30]. P-700 was assayed spectrophotometrically at 702 nm with a Hitachi 356 dual-wavelength spectrophotometer [31]. Mn and Ca^{2+} contents were determined with a Shimadzu atomic absorption spectrophotometer (AA640-01) equipped with a graphite furnace atomizer (GFA-2). Contaminating Ca^{2+} was removed by the chelex 100 treatment for 30 min at a chlorophyll concentration of 100–150 μg/ml [13].

Polypeptide compositions were analyzed by SDS polyacrylamide gel electrophoresis using the buffer system of Laemmli [32]. Samples were treated with 8 M urea, 2.5% SDS and 5% β-mercaptoethanol and applied to a 10–15% acrylamide gradient gel containing 6 M urea. The gel was stained with Coomassie Brilliant Blue R-250 and scanned at 560 nm or photographed.

Oxygen evolution was measured with a Clark-type oxygen electrode at 27 °C. Samples were suspended in the MNM medium containing 0.4 M sucrose. For determination of Cl^--requirement, the PS II preparations were washed twice with a Cl^--free medium containing 40 mM Mes/NaOH (pH 6.5) and 0.4 M sucrose and oxygen evolution was determined in the same medium. The concentration of contaminating Cl^- in the medium was assumed as 0.3 mM [24]. Chlorophyll concentration was determined according to Arnon [33].

Results

Highly active oxygen-evolving PS II membranes have been prepared from rice thylakoid membranes with Triton X-100 [17]. However, the Triton method failed to yield an active PS II preparation from the thylakoid membranes of the rice chlorina mutant. No clear separation of the two photosystems was obtained in a wide range of Triton X-100 to chlorophyll weight ratios (2.5–50) examined. A PS II fraction competent in oxygen evolution was, however, prepared with β-octylglucoside from the mutant membranes.

Fig. 1 shows the absorption spectrum of the PS II fraction isolated from the rice mutant. As compared with the spectrum of the Triton-PS II membranes from the wild-type rice, a chlorophyll *b* band at 650 nm was missing and the maximum of the red band was slightly shifted to a shorter wavelength. Another notable feature was a low scattering level of the suspension, suggesting a smaller particle size of the mutant preparation as compared with the Triton preparation from the normal rice. The fluorescence emission spectrum of the mutant thylakoids determined at 77 K showed two bands at 685 and 695 nm, which are related to PS II, and a broad band at 720 nm, which arises from PS I antenna chlorophyll *a* (Fig. 2). The mutant PS II particles show a main

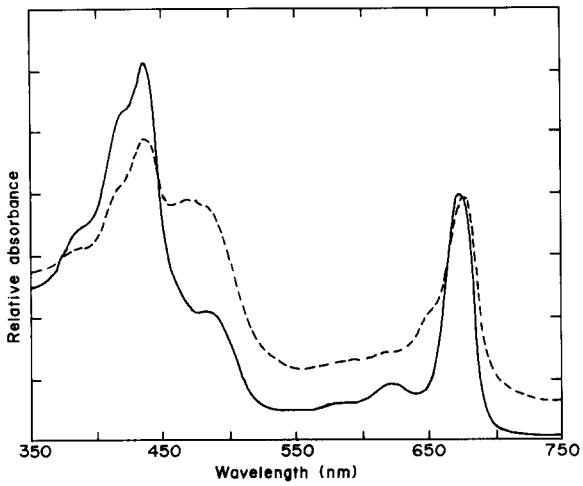

Fig. 1. Absorption spectra of the PS II preparations isolated from wild-type rice (— — —) and the chlorina mutant (———).

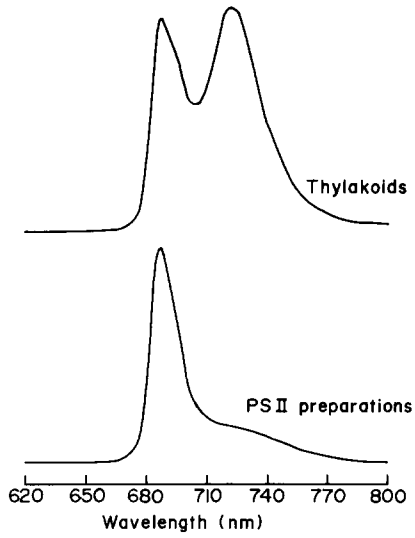

Fig. 2. Fluorescence emission spectra at 77 K of the thylakoid membranes and PS II particles from the rice mutant.

emission peak around 686 nm with a slight shoulder at 695 nm but no prominent band at 720 nm. This indicates that the preparation is essentially free from PS I.

The absence of PS I was also confirmed by SDS-gel electrophoresis of the mutant PS II preparations (Fig. 3). Lane 1 shows polypeptide bands resolved from the thylakoid membranes of the chlorina mutant. The slowest moving band of about 60 kDa is the chlorophyll-carrying large subunit(s) of the PS I reaction center complex. The mutant is relatively less abundant in the PS I proteins as compared with the wild type rice [27]. Note that the 60 kDa protein was absent from the mutant PS II preparation (lane 2).

The PS II preparation showed an intense band of about 57 kDa, which was identified as the large subunit of ribulose-1,5-bisphosphate carboxylase with a specific antibody raised against the rice enzyme (data not shown). The enzyme subunit appears to rebind preferentially to the PS II particles during preparation because relative abundance of the 57 kDa subunit in the isolated particles was larger than that in the thylakoid membranes. PS II membrane preparations isolated from wild type rice were not associated with the enzyme subunit (lane 5). Two bands, which migrated at 47 and 43 kDa regions, are the antenna chlorophyll-carrying proteins of the PS II reaction center

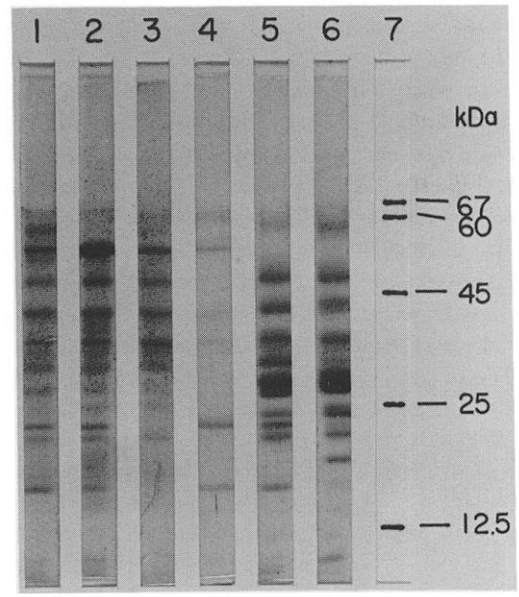

Fig. 3. Polypeptide compositions analyzed by SDS polyacrylamide gel electrophoresis. Lane 1, thylakoid membranes of the rice mutant; lane 2, PS II particles from the rice mutant; lane 3, NaCl-washed PS II particles of the rice mutant; lane 4, NaCl extract of the mutant PS II particles; lane 5, PS II membranes from the wild-type rice; lane 6, PS II membranes from spinach, and molecular markers: bovine serum albumin (67 kDa), catalase (60 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa) and cytochrome (12.5 kDa).

complexes and a 33 kDa band is the extrinsic protein essential for the water oxidation [19,20,34]. The mutant thylakoid and PS II preparations largely lacked LHC II apoproteins of 26–29 kDa, which were present abundantly in the PS II membranes from the wild-type rice and spinach (lane 6). A 25 kDa band found in the mutant preparations may be an extraneous protein adsorbed or a proteolytic fragment produced during the preparation because the band was not detected in the mutant thylakoid membranes. A 23 kDa band was present in the PS II preparations from normal plants but not in the mutant preparations. Presumably, the band corresponds to the apoprotein of CP24, a chlorophyll *a/b* protein of PS II [35]. The two proteins of 21 and 15 kDa were solubilized by washing with 1.5 M NaCl (lanes 3 and 4). This indicates that the two proteins are extrinsic proteins involved in oxygen evolution [19,20], although their molecular masses are significantly smaller than the corresponding 23 and 17 kDa proteins of spinach preparations. The abundances of 33, 21 and 15 kDa extrinsic proteins in the mutant PS II preparations relative to those in the mutant thylakoid membranes were shown in Table I. The detergent-treatment caused no significant loss of the 33 kDa protein and about two thirds of the 21 kDa protein were still associated with the PS II preparations. However, a large fraction of the 15 kDa protein was solubilized during the preparation. The content of the 15 kDa protein was somewhat variable with preparations.

The above experiments indicate that the mutant PS II particles contain all the major subunit polypeptides of Triton PS II preparations from normal higher plants, which are directly or indirectly related to PS II electron transport. However, there are notable differences in the abundance of important functional components between the two PS II preparations (Table II). Due to the absence of LHC II, the mutant preparation has a smaller antenna size: there is one Q_A, the first quinone acceptor of PS II, per 53 chlorophyll, as compared to Triton-preparations from normal rice, which have the chlorophyll to Q_A ratio of about 200 [17]. Preliminary experiments failed to detect CP29, an antenna chlorophyll-protein of PS II [36], in the mutant preparations. Importantly, the chlorophyll to Ca^{2+} ratio was 56, indicating that the mutant preparations had only one Ca^{2+} per Q_A. Because Triton PS II membranes isolated from normal rice have two Ca^{2+} [17], this means that one of the two Ca^{2+} associated with PS II is solubilized by the β-octylglucoside treatment. On the other hand, the abundance of Mn in the mutant PS II particles is comparable to that of the Triton preparations [21]. The chlorophyll to Mn ratio of 13 indicates that there are 4.1 Mn per PS II.

Inspite of the diminished Ca^{2+} content, the mutant PS II particles were found to evolve oxygen at substantial rates. Table III shows rates of oxygen evolution determined in the presence of various electron acceptors. The highest rates of 1000 μmol O_2 per mg Chl per h were obtained with phenyl- and 2,6-dichloro-*p*-benzoquinone, whereas ferricyanide was a poor electron acceptor. Oxygen evolution with phenyl-*p*-benzoquinone was completely inhibited in the presence of 10 μM DCMU.

In the experiments shown in Table III, oxygen evolution was determined in the presence of 20 mM Cl^- and 5 mM Ca^{2+}. The PS II preparations, which are largely deficient in the 15 kDa extrinsic protein, exhibited a strong Cl^- requirement (see

TABLE I

RELATIVE ABUNDANCES OF THREE EXTRINSIC PROTEINS IN OXYGEN-EVOLVING PS II PARTICLES FROM THE RICE MUTANT

Peak areas of the three proteins in the densitograms were determined with the 47 kDa protein as a reference. Abundance of each protein in the thylakoid membranes was taken as 100%.

Proteins (kDa)	Percent abundance (%)
33	107 ± 15
21	64 ± 0.7
15	20 ± 11

TABLE II

RELATIVE ABUNDANCES OF Q_A, Mn AND Ca IN THE MUTANT PS II PARTICLES

Components	mol Chl/mol
Q_A	53 ± 3.0
Ca	56 ± 7.9
Mn	13 ± 1.7

TABLE III

OXYGEN-EVOLVING RATES OF THE MUTANT PS II PARTICLES IN THE PRESENCE OF VARIOUS ELECTRON ACCEPTORS

The reaction mixtures contained 40 mM Mes/NaOH (pH 6.5), 10 mM NaCl, 5 mM $CaCl_2$, 0.4 M sucrose, PS II preparations (2–3 μg chlorophyll/ml) and indicated amount of an electron acceptor.

Electron acceptors	Concentrations (mM)	Rates of oxygen evolution (μmol O_2 per mg Chl per h)
Phenyl-*p*-benzoquinone	0.4	1100
2,6-Dichloro-*p*-benzoquinone	0.4	1070
2,6-Dimethyl-*p*-benzoquinone	1.0	470
2,5-Dimethyl-*p*-benzoquinone	1.0	390
Benzoquinone	1.0	340
Ferricyanide	1.0	270
2,6-Dichlorophenolindophenol	0.2	200
Phenyl-*p*-benzoquinone	0.4	
Phenyl-*p*-benzoquinone plus DCMU	0.01	0

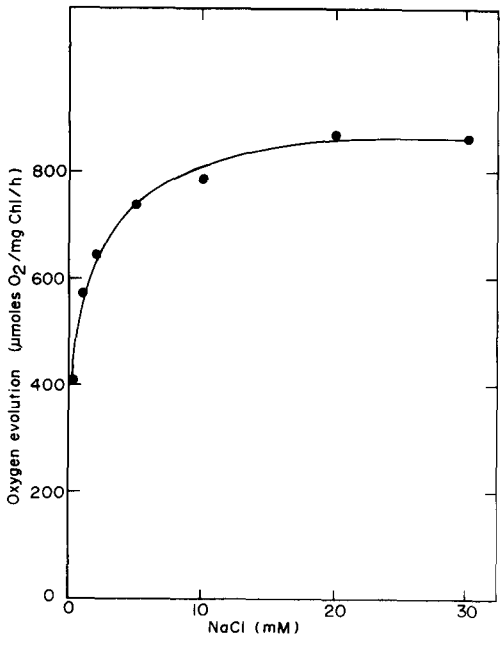

Fig. 4. Dependence on chloride concentration of oxygen evolution in the PS II particles isolated from the rice mutant. Reaction mixtures contained 40 mM Mes/NaOH (pH 6.5), 0.4 M sucrose, 1 mM phenyl-*p*-benzoquinone, PS II preparations (2–3 μg chlorophyll/ml) and indicated concentrations of NaCl.

TABLE IV

OXYGEN-EVOLVING ACTIVITY OF THE THYLAKOIDS AND PS II PARTICLES FROM THE RICE MUTANT

The 21 and 15 kDa proteins were extracted from the PS II membranes prepared from wild-type rice by inbucating with 1.5 M NaCl for 30 min at 0 °C and, after centrifugation at 35000 × *g* for 10 min, the supernatants were dialyzed against a large volume of 40 mM Mes/NaOH (pH 6.5) and 0.4 M sucrose for 9 h. The proteins were added to the assay medium at a molar ratio of two to one Q_A.

Additions	μmoles O_2 per mg Chl per h	
	Thylakoids	PS II particles
No additions	890	860
5 mM $CaCl_2$	910	1110
21 and 15 kDa proteins	–	1120

Refs. 37 and 38) and the maximum rate of oxygen evolution was obtained in the presence of about 20 mM Cl^- (Fig. 4). On the other hand, the PS II preparations evolved oxygen at a high rate in the absence of Ca^{2+}, although addition of 5 mM Ca^{2+} stimulated the activity by 30% (Table IV). The rate of oxygen evolution determined in the absence of exogenously added Ca^{2+} was comparable to that observed in the mutant thylakoids. The Ca^{2+} effect is related to the partial loss of the 21 kDa extrinsic protein during the preparation. In fact, addition of the 21 and 15 kDa proteins to the PS II particles enhanced oxygen evolution to a level similar to that attained with 5 mM Ca^{2+}. The activity of the reconstituted particles was insensitive to Ca^{2+}, whereas treatment of the preparations with 1.5 M NaCl created a strong Ca^{2+}-demand for oxygen evolution (data not shown).

Discussion

Isolation and properties of the mutant PS II preparations

The present work reports the isolation of an oxygen-evolving PS II fraction from a chlorophyll *b*-deficient mutant of rice, chlorina 9, with β-octylglucoside. Triton X-100, which has been used for preparation of PS II membranes from a chlorophyll *b*-less mutant of barley [39], failed to yield an active PS II preparation from the rice mutant. The isolation procedure used here is similar to that employed for preparation of the oxygen-

evolving PS II fraction from the cyanobacterium, *Synechococcus* sp. [6]. In both cases, β-octylglucoside preferentially solubilized PS II, leaving PS I in the membraneous fractions precipitated at the bottom of tubes. This indicates that the organization of the two photosystems in the mutant thylakoid membranes has some resemblance to that in the cyanobacterial membranes, which also lack LHC II.

The mutant preparation is essentially free from PS I, but contain the major proteins of the Triton-PS II membranes isolated from the wild-type rice, except the apoproteins of LHC II in the 26–29 kDa region. Unexpectedly, however, a large amount of ribulose-1,5-bisphosphate carboxylase was found in the mutant PS II preparations. Because the mutant thylakoids were less abundant in the enzyme, the enzyme protein solubilized with β-octylglucoside must have bound preferentially to the PS II preparations. The PS II membranes prepared from the wild-type rice had no affinity for the enzyme protein. Thus, the exposure of the binding sites for the enzyme protein in the mutant preparation may be related to the absence of LHC II.

The highest rates of oxygen evolution was obtained with phenyl- and dichloro-*p*-benzoquinone as electron acceptors, whereas ferricyanide supported only a low rate of oxygen evolution in the mutant PS II preparations. A similar acceptor dependency of oxygen evolution has been observed in PS II preparations from wheat [24] and *Synechococcus* which contain Q_B (Ref. 40; see also Takahashi, Y. and Katoh, S., unpublished results), but not in highly purified oxygen-evolving complexes prepared from the wheat PS II membrane preparations, which apparently lack the functional Q_B [24]. This, together with its strong sensitivity to DCMU, indicates that the mutant preparation still retains an intact acceptor system of PS II involving two bound plastoquinones, Q_A and Q_B.

Number of Ca^{2+} involved in PS II electron transport

The most important finding obtained in the present work is that the mutant PS II particles, which contain only one bound Ca^{2+} per PS II, show high rates of oxygen evolution in the absence of exogenously added Ca^{2+}. The rate of oxygen evolution exceeded 1000 μmol O_2 per mg Chl per h when the preparations are supplemented with the 21 and 15 kDa proteins. Because the Ca^{2+} abundance of the mutant preparations is half that of the Triton PS II membranes from normal higher plants, the results indicate that the Ca^{2+} that is preferentially solubilized by β-octylglucoside is not essential for PS II electron transport. The finding is consistent with the previous observation that the Ca^{2+} content of spinach PS II preparation can be halved by the chelex treatment in the presence of digitonin without a significant inactivation of oxygen evolution [16].

An alternative explanation for the Ca^{2+} to PS II ratio of one in the rice mutant preparations would be that β-octylglucoside extracts two Ca^{2+} from about 50% of the PS II particles, while leaving the Ca^{2+} abundance of another 50% totally unaffected. Thus, oxygen evolution of the preparations, which is attributed to the unaffected half of the PS II particles, is rather insensitive to Ca^{2+}. However, the following observations strongly argue against this possibility.

The maximum activity of the mutant preparation is 1100 μmol O_2 per mg chl per h. If chlorophyll is assumed to distribute evenly between PS I and PS II in the mutant membranes, which show oxygen evolution rates of about 900 μmol O_2 per mg Chl per h, the activity of the PS II particles is about 60% of that in the membranes on the basis of the PS II reaction center. However, PS II is considerably enriched relative to PS I in the chlorophyll *b*-less mutant to compensate an imbalance, caused by the absence of LHC II, in the distribution of light energy between the two photosystems [26,27]. Preliminary experiments showed that the mutant thylakoid membranes have one Q_A per 90 chlorophyll, four Mn atoms per 73 chlorophyll and one P-700 per about 300 chlorophyll. The rate of oxygen evolution in the PS II particles is 73% of that in the thylakoid membranes on the basis of Q_A. A larger value of 88% is obtained when Mn is used for the estimation of PS II reaction center. If we assume that the number of chlorophyll associated with PS I reaction center complexes without LHC I is 100 [41,42], the activity of the PS II particles corresponds to 82% of that in the thylakoid membranes. Thus, the maximum activity of the PS II particles signifi-

cantly exceeds the half-maximum activity of the thylakoid membranes on the basis of PS II reaction center. This is inconsistent with the alternative explanation stated above and indicates that the loss of one Ca^{2+} causes only minor defects in PS II electron transport. A possibility remains, however, that the extracted Ca^{2+} may have some role in optimizing PS II electron transport because the recovery of the activity was not 100%.

Oxygen-evolving complexes, which also contain one Ca^{2+} per PS II, were prepared with β-octylglucoside [24]. The complexes show very low rates of oxygen evolution in the absence of Ca^{2+} but the activity amounting to 1500 μmol O_2 per mg Chl per h was obtained on addition of the metal cations [24]. This is again incompatible with the view that the detergent extracts two Ca^{2+} from half the PS II centers.

It has been suggested that the two Ca^{2+} present in spinach PS II membranes are dissimilar from each other in terms of binding affinity or environment surrounding the binding site [16]. The results obtained here, together with previous observations that the Ca^{2+} abundance of PS II membrane preparations is halved by treatments with various detergents [16,17] or at an acidic pH [23] support this proposal.

The extrinsic 23 kDa protein has been suggested to provide a high affinity site for Ca^{2+} or stabilize the Ca^{2+}-binding [4,10]. The previous observation that the solubilization of the 23 kDa protein from spinach PS II membranes by NaCl-wash is not accompanied by any significant loss of the bound Ca^{2+} is, however, incompatible with this postulation (Ref. 17, but see Ref. 14). Comparison of the two β-octylglucoside preparations containing one Ca^{2+} per PS II also indicates that there is no correlation between the Ca^{2+} and 21(23) kDa protein contents: the mutant preparations still retain two thirds of the 21 kDa protein, whereas the purified oxygen-evolving complexes have an extremely low level of the protein [24]. On the other hand, the content of 21(23) kDa protein is well related to the magnitude of Ca^{2+}-dependent stimulation of oxygen evolution. The activity of the purified complexes is stimulated by more than 10 times on addition of Ca^{2+} [24]. In contrast, the Ca^{2+} effect is much smaller in the mutant preparation and disappeared when reconstituted with the protein. These are consistent with the explanation that exogenously added Ca^{2+} restores the functional conformation of PS II preparatons, which is perturbed by removal of the 23 kDa protein.

It has been shown previously that only one bound Ca^{2+} is needed to promote high rates of oxygen evolution in the cyanobacterium, *Synechococcus* sp. [9,13]. This is no longer attributed to a peculiarity of the prokaryotic system because a substantial rate of oxygen evolution occurs in the presence of one bound Ca^{2+} in the higher plant preparations. There is evidence suggesting that the bound Ca^{2+} has a role in electron transport from Z to P-680 in the cyanobacteria [7]. Experiments are in progress to examine whether or not the Ca^{2+} tightly bound to the mutant PS II preparation functions in the same region of electron transport.

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