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Immobilization of the three extrinsic proteins in spinach oxygen-evolving Photosystem II membranes: roles of the proteins in stabilization of binding of Mn and Ca^{2+}

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Abstract

The three extrinsic proteins of 33, 23 and 17 kDa were immobilized by treating oxygen-evolving Photosystem II (PS II) membranes with a zero-length crosslinker, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The 33 kDa and 17 kDa proteins were totally immobilized at 1% and 2% EDC, respectively. The 23 kDa protein was also immobilized in parallel to the 17 kDa protein at low concentrations of EDC, but a small fraction of the 23 kDa protein remained uncrosslinked at 3% EDC. Immobilization of the three proteins had no or only a minor inhibitory effect on oxygen evolution. The amount of Mn that was gradually released from urea/NaCl-washed PS II membranes was reduced from two to one per PS II by immobilization of the 33 kDa protein and further to a lower level by crosslinking of the 23 and 17 kDa proteins. Stabilization of Mn by the latter two proteins was also suggested by release of a small amount of Mn from NaCl-washed membranes during prolonged incubation at 0°C. Reductant-induced inactivation of oxygen evolution in urea/NaCl-washed membranes, which is ascribed to reduction and subsequent release of Mn, was suppressed by more than 50% by immobilization of the 33 kDa protein and further to a small extent by that of the 23 and 17 kDa proteins. Immobilization of the 23 kDa (and 17 kDa) protein(s) rendered oxygen evolution significantly resistant to treatment with 1.5 M NaCl and at low pH levels. Thus, binding of a functional Ca^{2+} is stabilized by covalent binding of the protein against not only salt- but also acid-extraction.

Key words: Immobilization; Crosslinking; Oxygen evolution; Oxygen stabilization; Extrinsic protein

1. Introduction

The three extrinsic proteins of 33, 23 and 17 kDa of Photosystem II (PS II) associated with lumenal surface of the thylakoid membranes play important roles in photosynthetic oxygen evolution (see for reviews [1–3]). The 33 kDa protein is considered to be essential for oxygen evolution [1–6]. The activity is strongly suppressed by removal of the protein with high concentrations of divalent metal cations [7] or urea plus NaCl [8,9] and partly restored by rebinding by the protein (but see [10–12]). The removal of the protein leads to gradual liberation of two out of four Mn per PS II present in the oxygen-evolving PS II membranes at 0°C [4]. It was suggested, therefore, that the protein stabilizes the binding or the functional conformation of the Mn cluster, which plays a central role in water oxidation [4–6]. Treatment of PS II membranes with 1.0–2.0 M NaCl results in liberation of the 17 and 23 kDa proteins, concomitant with inactivation of oxygen evolution [13–17]. The 23 kDa protein has a role to protect a functional Ca^{2+} and the lost activity of NaCl-washed PS II membranes is restored by addition of Ca^{2+} [17–21]. The Mn-cluster becomes sensitive to destruction with several reductants when the 17 and 23 kDa proteins [22] or all three proteins [23] are extracted. Thus, the proteins also have a role to shield Mn from reductants.

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Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; PS II, Photosystem II; LHC II, light-harvesting chlorophyll *a*/*b* protein of PS II; MES, 4-morpholineethanesulfonic acid; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

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Recently, we have shown that the 33 kDa protein is totally immobilized with a zero-length crosslinker, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), without any effects on oxygen evolution [24]. The one-to-one stoichiometry between the protein and the chlorophyll-carrying 47 kDa protein was demonstrated [24]. In addition, the oxygen-evolving activity was significantly stabilized by the immobilization of the 33 kDa protein against $CaCl_2$- or urea/NaCl-wash.

In the present study, we report that the two extrinsic proteins of 17 and 23 kDa are also immobilized at high concentrations of EDC with no or only a minor effect on oxygen evolution. Effects of immobilization of the three extrinsic proteins on the stability of oxygen evolution and binding of Mn and Ca^{2+} were investigated.

2. Materials and methods

Oxygen-evolving PS II membranes were prepared from spinach as in [25], except that a Triton X-100/chlorophyll ratio of 14:1 was used. The membranes were washed once and suspended in a medium containing 40 mM MES-NaOH (pH 6.5), 0.4 M sucrose, 5 mM $MgCl_2$ and 10 mM NaCl (medium A).

For crosslinking, indicated amounts of EDC were added to suspensions of PS II membranes (1 mg Chl/ml) and incubated for 10 min at room temperature. Then, the suspension was diluted with about 30 volumes of medium A and the treated membranes were quickly pelleted by centrifugation. The concentration of EDC required for the total crosslinking of the 33 kDa protein varied due to a highly deliquescent property of the crosslinker [24]. In the present study, precautions were taken so that all the data can be compared with each other in terms of the crosslinker concentration.

For removal of the three extrinsic proteins, PS II membranes were treated with 2.6 M urea and 0.2 M NaCl for 30 min at 0°C [8]. Protein-depleted membranes were washed once with and suspended in medium A for assay of oxygen evolution. The 17 and 23 kDa proteins were extracted by NaCl-wash as described in [15] with slight modifications. PS II membranes (0.5 mg Chl/ml) were incubated with indicated concentrations of NaCl for 30 min at 0°C in room light. Low pH-treatment was carried out by the procedure described by Ono and Inoue [26]. NaCl- and low pH-treated membranes were precipitated by centrifugation at $35\,000 \times g$ for 10 min, washed once with and suspended in a medium containing 40 mM MES-NaOH (pH 6.5), 0.4 M sucrose, 20 mM NaCl and 0.5 mM EDTA.

Polypeptides were analyzed by polyacrylamide gel electrophoresis. Samples were treated with 5% lithium dodecylsulfate and 60 mM dithiothreitol for 30 min at room temperature and applied to gels containing 6 M urea according to the method of Laemmli [27]. Acrylamide concentrations were 4.5% for the stacking and 11.5% for the resolving gels. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 and photographed or scanned at 555 nm with a Shimadzu CS-910 chromatoscanner.

Oxygen evolution was measured with a Clark-type oxygen electrode at 25°C. Electron acceptor used was 0.4 mM phenyl-*p*-benzoquinone. Actinic light of a saturating intensity (5 mmol quanta/m^2/s) passed through a Toshiba Y-50 filter. For determination of Mn released from NaCl- or urea/NaCl-washed PS II membranes, membrane suspensions were centrifuged at $35\,000 \times g$ for 10 min and Mn remaining in the supernatant was assayed with a Hitachi polarized Zeeman atomic absorption spectrophotometer (Z-8000). Amounts of Mn per PS II were estimated by assuming the antenna size of PS II as 220 chlorophyll. All experiments were performed at least three times to check reproducibility.

3. Results and discussion

Immobilization of the three extrinsic proteins

Fig. 1A shows proteins resolved by gel electrophoresis from oxygen-evolving PS II membranes treated with different concentrations of EDC. The chlorophyll-carrying 47 kDa protein of PS II reaction center complex (CP 47) and the extrinsic 33 kDa protein preferentially disappeared at low concentrations of EDC. The previous work showed that the two proteins are totally crosslinked to each other at or below 1.0% EDC, yielding a one-to-one crosslinked product at a position marked A [24]. Another distinct product that appeared (labeled B) is formed by the crosslinked apoproteins of LHC II [24].

Crosslinking of other proteins was observed in the present study, where higher concentrations of the crosslinker were used. The 17 kDa protein mostly disappeared when EDC concentration was raised above 1.5%. The band intensity of the 23 kDa protein also decreased with increasing concentration of the crosslinker. However, amounts of the 23 kDa protein remaining uncrosslinked could not be accurately determined due to comigration of another protein. The amounts of non-crosslinked proteins were, therefore, determined by extracting them with 2.6 M urea plus 0.2 M NaCl [9,24]. Fig. 1B shows that the 17 kDa protein was totally immobilized at 2% EDC. The 23 kDa protein was also crosslinked, but a small population of the protein remained unimmobilized even at the highest concentration of EDC used. EDC is a zero-length crosslinker which reacts with carboxyl and amino groups to generate a peptide bond between two proteins. The

result suggests, therefore, that salt-bridging is involved in the binding of the two extrinsic proteins to their partner proteins. No crosslinked products of the two extrinsic proteins were detected. Thus, the 17 and 23 kDa proteins were immobilized by crosslinking with some intrinsic membrane proteins or with the immobilized 33 kDa protein.

Many crosslinked products appeared in the high molecular mass region of the gel. For identification of a protein with which the 23 or 17 kDa protein was crosslinked, immunoblotting with the antisera raised against the ten major proteins of PS II membranes (CP

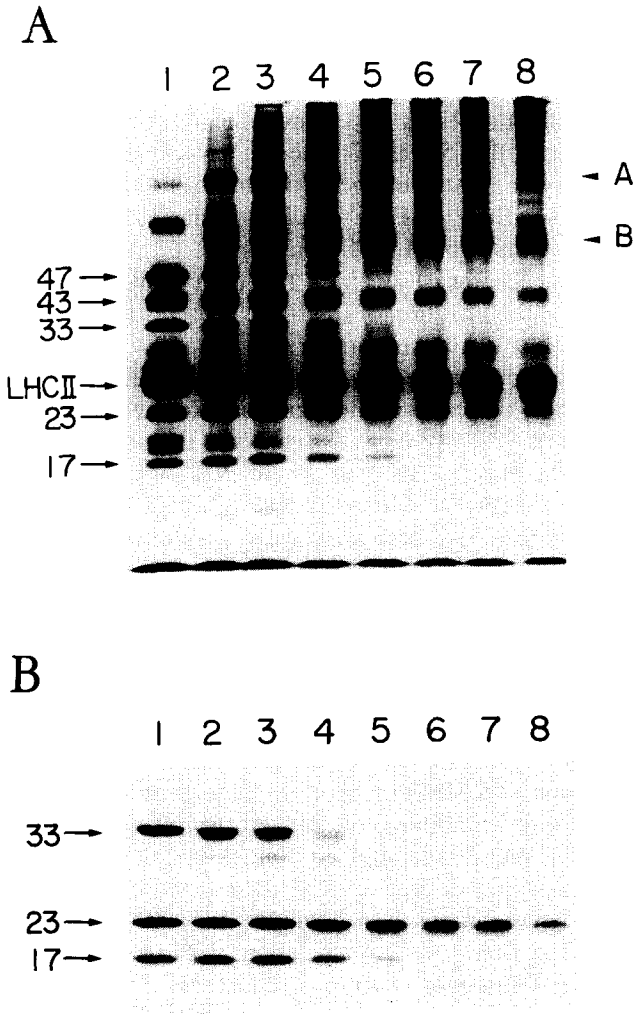

Fig. 1. Effects of crosslinking treatment with EDC on polypeptide patterns of oxygen-evolving PS II membranes resolved by gel electrophoresis (A) and the three extrinsic proteins extracted from EDC-treated PS II membranes by urea/NaCl-wash (B). PS II membranes were treated with EDC at final concentrations of 0% (lane 1), 0.1% (lane 2), 0.2% (lane 3), 0.5% (lane 4), 1.0% (lane 5), 1.5% (lane 6), 2.0% (lane 7) and 3.0% (lane 8) for 10 min at room temperature. Numbers on the left indicate apparent molecular masses (kDa) of bands resolved. (B) PS II membranes treated with EDC as above were incubated with 2.6 M urea plus 0.2 M NaCl for 30 min at O°C and, after centrifugation, the supernatants obtained were applied to SDS gel electrophoresis.

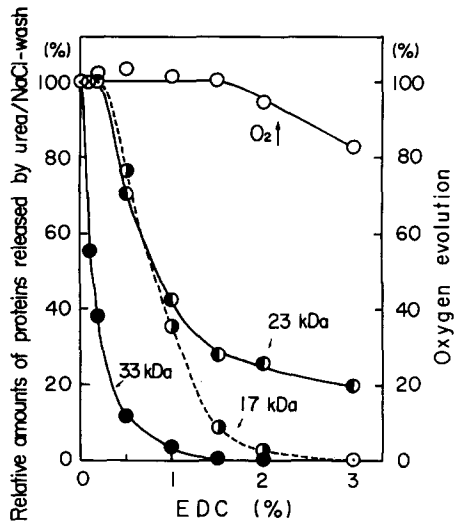

Fig. 2. The oxygen-evolving activity and percentages of the three extrinsic proteins unimmobilized in PS II membranes treated with different concentrations of EDC. Amounts of the extrinsic proteins solubilized by urea/NaCl-wash were estimated by measuring peak areas of protein bands in densitometric tracings, taking the peak area of each protein solubilized from non-crosslinked PS II membranes as 100%. Rate of oxygen evolution of untreated PS II membranes (100%) was 605 μmol O_2/(mg Chl) per h.

47, CP 43, the D1 and D2 proteins, the LHC II apoproteins, the extrinsic 33, 23 and 17 kDa proteins, the large subunit of cytochrome *b*-559 and the *psb*I gene product [24,28]) was carried out. However, except for the products A and B, none of the CBB-stained product bands reacted appreciably with the antisera examined (data not shown). This result implies that EDC-treatment diminishes immunological reactivity of proteins through modification of carboxyl groups [28].

The previous study showed that the extrinsic 33 kDa protein could be immobilized without significant inhibition of the oxygen-evolving activity [24]. Experiments were carried out to examine whether immobilization of the 17 and 23 kDa proteins affects oxygen evolution. Fig. 2 shows rates of oxygen evolution in PS II membranes treated with different concentrations of EDC. For the comparison, amounts of the three extrinsic proteins remaining uncrosslinked were determined. The 33 kDa and 17 kDa proteins were almost completely immobilized at 1.0% and 2.0% EDC, respectively, whereas about 20% of the 23 kDa protein remained uncrosslinked even at 3.0% EDC. Note that the rate of oxygen evolution was little affected by treatment with 1.5% EDC, where the 33 kDa protein was totally and the 17 and 23 kDa proteins were largely immobilized. We conclude, therefore, that crosslinking of the three proteins has no inhibitory effect on the oxygen-evolving activity. However, the activity gradually decreased at higher concentrations of the crosslinker. This may be ascribed to additional effects of EDC such as inter- or intra-molecular crosslinking

of other functional proteins or chemical modification of carboxyl groups.

Stabilization of Mn by immobilization of the extrinsic proteins

Having succeeded in immobilization of the three extrinsic proteins with no or a small loss of the activity, we proceeded to investigate the roles of the proteins in stabilizing functional components of oxygen evolution. We first studied effects of immobilization of the three extrinsic proteins on the stability of Mn which plays an important role in the oxidation of water. Removal of the three proteins by urea/NaCl-wash induced gradual release of Mn from PS II membranes, resulting in loss of two out of the four bound Mn during 2 days at 0°C in the dark as described in [8,9] (Fig. 3). The loss of Mn was nearly halved by treatment of PS II membranes with 0.5% EDC prior to urea/NaCl-wash (curve b). This concentration of the crosslinker immobilized 90% of the 33 kDa protein and 20–30% of the 17 and 23 kDa proteins (see Fig. 2). Thus, stabilization of one of the two labile Mn is ascribed to immobilization of the 33 kDa protein. Crosslinking of the PS II membranes with 3.0% EDC further reduced the amount of Mn released during prolonged incubation (curve c). This indicates that the remaining labile Mn is stabilized by the 17 and 23 kDa proteins, or some other proteins, crosslinked at this concentration of the crosslinker.

The results presented in Table 1 favor the view that immobilization of the 17 and 23 kDa proteins is responsible for the protection of Mn. When the two proteins had been removed by NaCl-wash, a small amount of Mn was gradually solubilized during incubation at 0°C for 4 days. The loss of Mn is related to removal of the two proteins because unwashed membranes lost essentially no Mn during the same period of incubation time, and immobilization of the 33 kDa protein with 0.5% EDC failed to abolish the release of Mn. Note that treatment of unwashed PS II membranes with 3.0% EDC resulted in destabilization of a small amount of Mn. The incomplete protection of Mn against NaCl-wash (Table 1) or urea/NaCl-wash (Fig. 3) by immobilization of the three proteins is ascribed to this effect of the crosslinker.

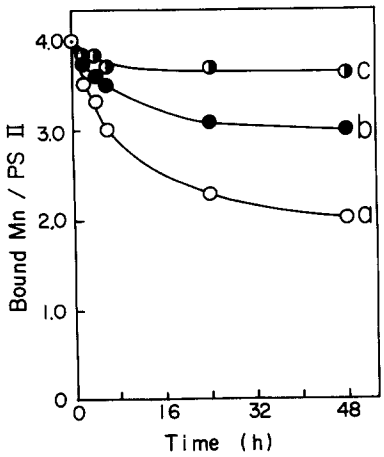

Fig. 3. Effects of immobilization of the three extrinsic proteins on release of Mn from urea/NaCl-washed PS II membranes. PS II membranes which had been treated with 0% (curve a), 0.5% (curve b) or 3.0% (curve c) EDC were washed with 2.6 M urea plus 0.2 M NaCl and left at 0°C in the dark.

Table 1
Release of Mn from NaCl-washed PS II membranes

EDC (%)	Treatment	Mn released per PS II	
		2 days	4 days
0	–	0.00	0.03
0	NaCl-wash	0.10	0.32
0.5	–	0.00	0.00
0.5	NaCl-wash	0.08	0.20
3.0	–	0.10	0.27
3.0	NaCl-wash	0.14	0.30

PS II membranes (0.5 mg Chl/ml), either non-crosslinked or crosslinked with 0.5% or 3.0% EDC, were treated with 1.5 M NaCl, washed once with and suspended in medium A (1 mg Chl/ml) and then left for indicated periods of time at 0°C in the dark.

Mn is rapidly released when PS II membranes depleted of the extrinsic proteins are exposed to reducing reagents [22,23]. It was suggested that the extrinsic proteins have a role to shield Mn from reductants which destabilize Mn by reducing Mn(III)/Mn(IV) to Mn(II) [22,23]. There is, however, a discrepancy as to which of the three extrinsic proteins plays a major role in protecting Mn. Ghanotakis et al. [22] reported that removal of the 23 and 17 kDa proteins renders up to 80% of Mn sensitive to exogenous reductants. However, Tamura et al. [23] showed that further release of the 33 kDa protein is needed for strong inactivation of oxygen evolution by reductants. Table 2 shows that addition of TMPD or hydroquinone resulted in only a moderate inhibition of oxygen evolution in NaCl-washed membranes but an almost complete inhibition of the activity in urea/NaCl-washed membranes. When PS II membranes had been treated with 0.5% EDC prior to urea/NaCl-wash, about 50% of the activity survived treatment with the reductants. Further crosslinking of the 17 and 23 kDa proteins with 3.0% EDC increased the stability of oxygen evolution only to small extents both in NaCl- and urea/NaCl-washed membranes. It is concluded from these results that the 33 kDa protein is a dominant factor to protect the Mn cluster from attack by exogenous reductants.

Stabilization of Ca^{2+}-binding by immobilization of the 23 kDa protein

Inactivation of oxygen evolution by NaCl-wash is related to extraction of Ca^{2+} and the 23 kDa protein is

Table 2
Effects of immobilization of the three extrinsic proteins on reductant-induced inactivation of oxygen evolution in NaCl- and urea/NaCl-washed PS II membranes

EDC (%)	Reductants	Rates of oxygen evolution (μmol O_2/(mg Chl)/h)		
		– (%)	NaCl-washed (%)	Urea/NaCl-washed (%)
0	–	537 (100)	457 (100)	114 (100)
	TMPD	526 (98)	315 (69)	4 (4)
	HQ	516 (96)	274 (60)	10 (9)
0.5	–	540 (100)	459 (100)	315 (100)
	TMPD	513 (95)	324 (71)	176 (56)
	HQ	497 (92)	315 (69)	158 (50)
3.0	–	464 (100)	408 (100)	399 (100)
	TMPD	404 (87)	306 (75)	263 (66)
	HQ	394 (85)	286 (70)	215 (54)

NaCl- or urea/NaCl-washed membranes were suspended in medium A containing 1 mM TMPD or 1 mM hydrobenzoquinone (HQ) (1 mg Chl/ml). After incubation for 60 min at 0°C in the dark, 20 μl of the sample suspension was added to 2 ml of medium A that contained 0.4 mM phenyl-*p*-benzoquinone and 50 mM $CaCl_2$ for assay of oxygen evolution.

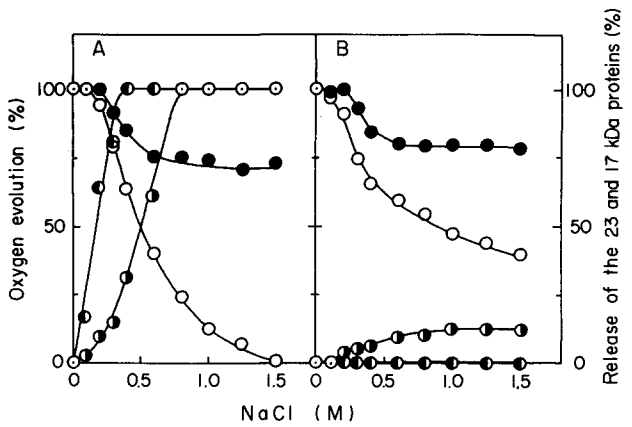

Fig. 4. Stabilization of oxygen evolution against NaCl-wash by immobilization of the extrinsic 23 and 17 kDa proteins. PS II membranes (0.5 mg Chl/ml), either non-crosslinked (A) or crosslinked with 3.0% EDC (B), were incubated with indicated concentrations of NaCl for 30 min at 0°C in room light. ○ and ●, rates of oxygen evolution determined in the absence and presence of 5 mM $CaCl_2$, respectively; ◐ and ◑, the 17 kDa and 23 kDa proteins solubilized by NaCl-wash, respectively. The 100% activities of oxygen evolution determined in the absence and presence of $CaCl_2$ were 530 and 558 μmol O_2/(mg Chl)/h for non-crosslinked membranes, and 461 and 521 μmol O_2/(mg Chl)/h for crosslinked membranes, respectively.

considered to protect Ca^{2+} [17,29–34]. A recent study showed that one Ca^{2+} located in the PS II reaction center core complex functions in oxygen evolution and the activity is lost when the Ca^{2+} is dissociated from its binding site by the action of light in the absence of the 23 kDa protein [35]. Experiments were carried out to examine whether immobilization of the 23 kDa protein stabilizes the oxygen-evolving activity against NaCl-wash. When non-crosslinked PS II membranes were incubated with various concentrations of NaCl in room light for 30 min, the 17 and 23 kDa proteins were totally solubilized at 0.4 and 0.8 M NaCl, respectively, and the oxygen-evolving activity was completely inactivated at 1.5 M NaCl (Fig. 4A). When the 17 and 23 kDa proteins had been immobilized with 3.0% EDC, the activity became considerably resistant to NaCl-wash (Fig. 4B). The activity remaining after treatment with 1.5 M NaCl amounted to 50% of the activity restored by addition of 5 mM Ca^{2+}. However, taking into account the incomplete immobilization of the 23 kDa protein (Fig. 2), the extent of stabilization of the activity is larger than it appears. The results show that immobilization of the protein substantially stabilized binding of the functional Ca^{2+} against the salt-wash in the light.

A brief treatment of PS II membranes at pH 3.0 solubilizes one Ca^{2+} [26,34]. The extrinsic proteins are also solubilized at pH 3.0 but rebind to PS II membranes when the pH of the medium is raised to pH 6.5 [36,37]. A question arises, therefore, as to whether dissociation of the 23 kDa protein is involved in acid-extraction of Ca^{2+}. Table 3 shows that immobilization of the three extrinsic proteins with 3.0% EDC resulted in stabilization of a small but significant activity against treatment at pH 3.0. The rate of oxygen evolution remaining after 5 min of treatment at pH 3.5 was increased by the protein immobilization from 10% to 42% of that restored by addition of 50 mM Ca^{2+}. Crosslinking of the 33 kDa protein alone had essentially no protecting effect (not shown). The results suggest that the 23 kDa (and 17 kDa) protein(s) has a role to stabilize Ca^{2+} against acid-extraction so that release of Ca^{2+} is facilitated by removal of the protein.

In conclusion, the present study indicates that EDC is an excellent reagent for immobilization of the three

Table 3
Effects of immobilization of the extrinsic proteins on inactivation of oxygen evolution by low pH-treatments

Acid treatment	Rates of oxygen evolution (μmol O_2/(mg Chl) per h)			
	Non-crosslinked		Crosslinked with 3.0% EDC	
	$-Ca^{2+}$	+50 mM Ca^{2+}	$-Ca^{2+}$	+50 mM Ca^{2+}
–	653	602	600	580
pH 3.0	0	286	69	298
pH 3.5	38	366	154	365

PS II membranes, either non-crosslinked or crosslinked with 3.0% EDC, were suspended in 20 mM citrate-NaOH (pH 3.0 or 3.5), 0.4 M sucrose and 20 mM NaCl. After incubation for 5 min at 0°C in the dark, the suspension was diluted with about 30 volumes of a medium containing 40 mM MES-NaOH (pH 6.5), 0.4 M sucrose, 20 mM NaCl and 0.5 mM EDTA and, after incubation for 10 min at 0°C in the dark, the membranes were collected by centrifugation at 35000× *g* for 10 min.

extrinsic proteins. The three proteins are totally or mostly immobilized with this reagent with no or only a small inhibitory effect on oxygen evolution. Thus, functions of the three proteins in photosynthetic oxygen evolution could be analyzed by means of protein crosslinking. The results obtained confirm that the extrinsic 33 kDa protein plays an important role in stabilization of Mn. In addition, the 23 and 17 kDa proteins were found to significantly contribute to stabilization of Mn. There is evidence indicating that loss of the oxygen-evolving activity is correlated with loss of the two out of four Mn present per PS II [8,38]. Interestingly, the results in the present work suggest that one of the two labile Mn is stabilized by immobilization of the 33 kDa protein and another by crosslinking of the 23 and 17 kDa proteins. Thus, we expect that differential crosslinking of the proteins would provide important information on functions of the two labile Mn. Experiments along this line are in progress. Immobilization of the 23 and 17 kDa proteins rendered Ca^{2+} more resistant against NaCl-wash. Inhibition of oxygen evolution by low pH-treatment was significantly reduced by the protein crosslinking. Thus, immobilization of the extrinsic proteins is a useful technique to stabilize oxygen evolution, the most labile reaction of photosynthesis. The effect of the immobilization of the proteins on thermal inactivation of oxygen evolution will be described elsewhere.

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