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Total immobilization of the extrinsic 33 kDa protein in spinach Photosystem II membrane preparations. Protein stoichiometry and stabilization of oxygen evolution

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(1) Treatment of oxygen-evolving Photosystem II membrane fragments (PS II membranes) with a zero-length crosslinker, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) led to immobilization of all the extrinsic 33 kDa protein molecules without any significant effects on the oxygen-evolving activity and oscillation patterns of flash-induced oxygen evolution and thermoluminescence B band. (2) With increasing concentration of EDC, the chlorophyll-binding 47 kDa protein decreased in parallel with the 33 kDa protein, yielding a crosslinked product consisting of one each of the two proteins. The results, which indicate that the two proteins are present in equimolar amounts in PS II membranes, are consistent with the stoichiometry of one copy of the 33 kDa protein per PS II unit. (3) The total immobilization of the 33 kDa protein stabilized 40 to 60% of the oxygen-evolving activity against urea/NaCl-, $CaCl_2$- and $MgCl_2$-wash, which otherwise solubilize the three extrinsic proteins and strongly inactivate oxygen evolution. The result implies that extraction of the extrinsic proteins may not be the sole cause of the inactivation of oxygen evolution by these treatments. (4) The crosslinking of the 33 kDa protein with EDC had no protecting effect against Tris-, NH_2OH- and pH 9.0-treatments. However, the stability of oxygen evolution at alkaline pH levels was slightly but significantly increased by treatment of PS II membranes with dithiobis(succinimidylpropionate), which specifically modifies amino groups.

Introduction

Oxygen evolution is one of the most unstable reactions of photosynthesis and selectively inactivated by various treatments [1–8]. A promising technique to stabilize the oxygen-evolving activity is crosslinking of the thylakoid membranes or PS II preparations. Crosslinking of the thylakoids with glutaraldehyde resulted in a stabilization of oxygen evolution against heat treatment and Tris- or $Cd(NO_3)_2$-wash [9]. The Mn-binding and photochemical activities became significantly resistant to heat- or NH_2OH-treatment, but not to the Tris-wash, when the PS II membranes had been fixed with the crosslinker [10]. Glutaraldehyde fixation of chloroplast also suppressed S_2-dependent inactivation of oxygen evolution by Tris-wash [11]. However, glutaraldehyde treatment itself is inhibitory on oxygen evolution and provides little information as to which protein(s) crosslinked is responsible for the stabilization of the activity [9,10].

The three extrinsic proteins of 33, 23 and 17 kDa associated with the lumenal surface of the thylakoid membranes are required for optimal functioning of the

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; LDS, lithium laurylsulfate; CBB, Coomassie brilliant blue R-250; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DSP, dithiobis(succinimidylpropionate).

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oxygen-evolving machinery. The three proteins are present in equimolar amounts [12–14], but it is still disputed whether one copy or two copies of each of the proteins are associated with PS II unit [13,15]. Solubilization of the proteins is associated with partial or total inactivation of oxygen evolution. In particular, removal of the 33 kDa protein from PS II membrane preparations by treatments with high concentrations of $CaCl_2$, $MgCl_2$ [8] or urea plus NaCl [16] results in a strong inhibition of oxygen evolution and the lost activity was substantially restored by reconstitution of the protein to protein-depleted membranes [17]. The 33 kDa protein is also considered to stabilize Mn binding because extraction of the 33 kDa protein by $CaCl_2$- or urea/NaCl-wash induces a gradually liberation of two Mn per PS II [16–20].

Crosslinking of the 33 kDa protein with the 47 kDa chlorophyll-carrying protein of the PS II reaction center complexes has been reported [21–24]. The previous work showed that immobilization of the 33 kDa protein with DSP significantly stabilized oxygen evolution against urea/NaCl-wash or treatment at pH 9.0 [23]. However, only 15–20% of the 33 kDa protein could be immobilized in its functional state and intensive treatment with the crosslinker resulted in an inhibition of oxygen evolution. Another crosslinker EDC was used to partially crosslink the 33 kDa protein, but the effect of the crosslinking on oxygen evolution has not yet been determined [22].

In the present work, attempts were made to immobilize the 33 kDa protein more extensively while keeping the oxygen-evolving activity unimpaired. EDC was found to crosslink all the 33 kDa protein molecules present in spinach PS II membranes with the chlorophyll-carrying 47 kDa protein at the one to one ratio. Oxygen evolution and the S-state transition of PS II membranes with the 33 kDa protein totally immobilized were determined. Effects of the protein immobilization on the stability of oxygen evolution against various inhibitory treatments of PS II membranes were also investigated.

Materials and Methods

Oxygen-evolving PS II membranes were prepared from spinach chloroplasts with Triton X-100 according to Berthold et al. [25] with slight modifications described in Ref. 23. Removal of the extrinsic 23 and 17 kDa proteins or the 33, 23 and 17 kDa proteins was carried out by incubating PS II membranes with 1.5 M NaCl [26,27] or 2.6 M urea plus 0.2 M NaCl [16], respectively, for 30 min at 0 °C. For crosslinking, various concentrations of EDC were added to samples (1.0–1.5 mg chlorophyll per ml) suspended in 40 mM Mes-NaOH (pH 6.5), 0.4 M sucrose, 5 mM $MgCl_2$ and 10 mM NaCl (solution A). EDC was dissolved into solution A prior to each crosslinking experiment. After incubation for 10 min at room temperature, the crosslinking reaction was terminated by centrifugation after addition of 0.25 M sodium acetate or a large amount of solution A.

It is to be mentioned that concentrations of EDC shown in each figure legend are the apparent ones because the crosslinker is strongly deliquescent and unstable. Thus, the concentration of the crosslinker required for the total immobilization of the 33 kDa protein varied in the range of 0.1 to 1.0% in the present study, where several different batches of the crosslinker had been used.

For analysis of polypeptides, samples were treated with 10% LDS and 60 mM dithiothreitol for 30 min at room temperature and applied to slab gel electrophoresis according to the method of Laemmli [28]. Acrylamide concentrations were 4.5% for stacking gels and 8.5–13.5% for resolving gels. Both gels contained 6 M urea. After electrophoresis, gels were stained with CBB for proteins and photographed or scanned at 555 nm with a Shimadzu CS-910 chromatoscanner. For immunoblotting, proteins resolved by gel electrophoresis were electrotransferred to a nitrocellulose membrane and detected by enzyme-linked immunoblot analysis as described previously [24,29].

Oxygen evolution was measured at 25 °C with a Clark-type oxygen electrode as in Ref. 23 and 30. PS II membranes (10 μg chlorophyll per ml) were suspended in solution A containing 0.4 mM phenyl-*p*-benzoquinone as an electron acceptor and illuminated with saturating white light. Flash yield of oxygen was measured in the absence of artificial electron accpetor with a Joliot-type oxygen electrode under a series of Xe flashes (white, 4 μs; 2J) fired at a uniform interval of 1 s [31]. Thermoluminescence was measured as described in Ref. 32 and 33. Dark-adapted samples were excited with a saturating Xe flash at 25 °C and quickly frozen in liquid N_2 and thermoluminescence glow curves were recorded at a heating rate of about 0.8 C°/s.

Results

Total immobilization of the 33 kDa protein

Protein patterns of oxygen-evolving PS II membranes treated with different concentrations of EDC are shown in Fig. 1A. Among the protein bands resolved, the extrinsic 33 kDa protein and the apoprotein of CP47, a chlorophyll-carrying 47 kDa protein, were preferentially diminished with increasing concentration of the crosslinker. Several crosslinked products appeared in the high molecular mass regions. Two major product bands were labeled A and B. When crosslinked membranes were washed with the urea/NaCl couple, which is known to solubilize all the three extrinsic proteins of 33, 23 and 17 kDa proteins [16], there were

corresponding decreases in the amount of the 33 kDa protein liberated (Fig. 1B). Thus, the 33 kDa protein was immobilized. Two or three weak bands which appeared below the 33 kDa protein may be ascribed to modification of carboxyl groups with the crosslinker or intramolecular crosslinking which would affect mobility

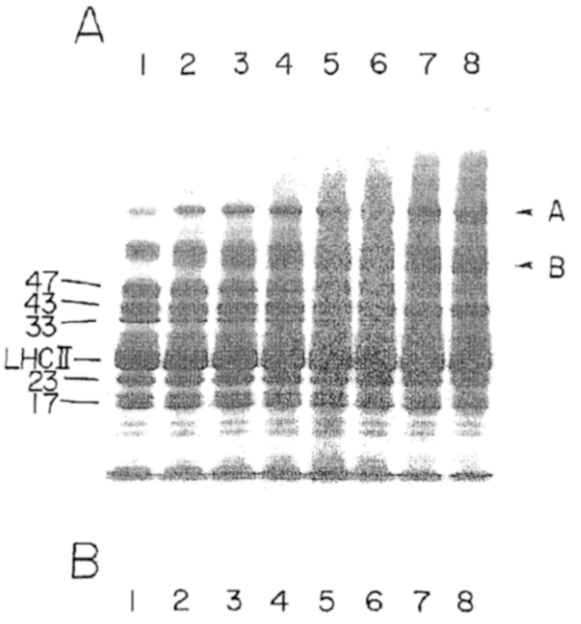

Fig. 1. (A) SDS gel electrophoresis of PS II membranes treated with various concentrations of EDC. PS II membranes were treated with EDC at final concentrations of 0% (lane 1), 0.05% (lane 2), 0.1% (lane 3), 0.15% (lane 4), 0.2% (lane 5), 0.3% (lane 6), 0.4% (lane 7) and 0.5% (lane 8) for 10 min at room temperature. After the crosslinking reaction had been terminated by the addition of 0.25 M sodium acetate, the membranes were incubated with 10% LDS and 60 mM dithiothreitol for 30 min, and then applied to 8.5–13.5% polyacrylamide gradient gel containing 6 M urea. (B) Proteins solubilized from PS II membranes crosslinked as above by treatment with 2.6 M urea and 0.2 M NaCl for 30 min at 0 °C. Gels were stained with CBB. Numbers on the left side indicate apparent molecular masses (kDa) of bands resolved.

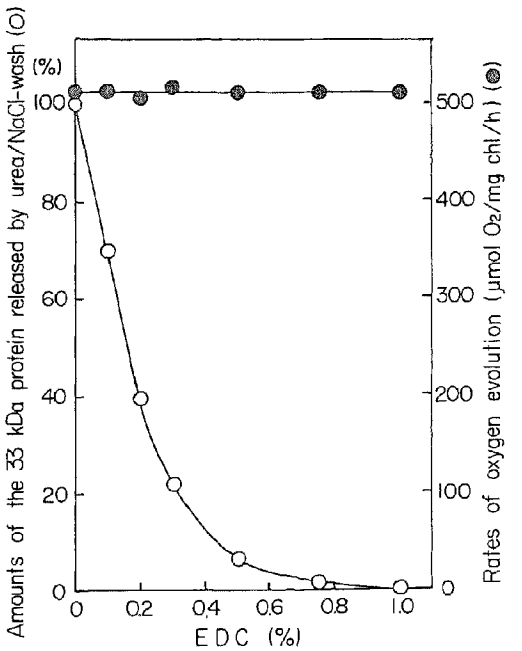

Fig. 2. Effects of immobilization of the 33 kDa protein on the oxygen-evolving activity of PS II membranes treated with various concentrations of EDC. Crosslinking of PS II membranes was carried out as described in Fig. 1, except that a wider range of EDC concentration was used. The uncrosslinked 33 kDa protein was extracted by urea/NaCl-wash, subjected to gel electrophoresis and quantified by measuring peak areas of the protein band in densitometric tracings. The band intensity of the 33 kDa protein released from untreated PS II membranes was taken as 100%.

of the protein. The 23 and 17 kDa proteins were immobilized to lesser extents.

Effects on oxygen evolution of the immobilization of the 33 kDa protein were examined (Fig. 2). In this experiment, a wider concentration range of EDC was used to ensure the total crosslinking of the protein. The results shown in Fig. 2 reveal two important features of the protein crosslinking. First, the amount of the 33 kDa protein released by urea/NaCl-wash decreased with increasing concentration of the crosslinker and became essentially zero at 1.0% EDC. Thus, all the 33 kDa protein molecules associated with PS II membranes could be immobilized. The second important feature is that the rate of oxygen evolution was not at all affected by the total crosslinking of the 33 kDa protein. This indicates that neither intermolecular nor intramolecular crosslinking of the 33 kDa protein affects the functional conformation of the protein.

The following results are also consistent with the normal functioning of the oxygen-evolving machinery in PS II membranes with the 33 kDa protein crosslinked. Production of one oxygen molecule from two molecules of water requires the storage of four

positive charges and, under repetitive flash excitations, flash yield of oxygen shows a characteristic four-step oscillation with maxima at the 3rd and 7th flashes [34,35]. Fig. 3A shows that EDC treatment of PS II membranes did not affect the oscillation of oxygen-yield. Oscillation pattern of the thermoluminescence B band, which arises from $S_2Q_B^-$ and $S_3Q_B^-$ charge recombination [36–38], was not significantly affected by the crosslinking treatment, either (Fig. 3B). EDC-treated membranes were sensitive to DCMU because the thermoluminescence B band was replaced by Q band, which arises from $S_2Q_A^-$ charge recombination [36,37] and oxygen evolution was strongly inhibited in the presence of the inhibitor (data not shown). These results indicate that the entire pathway of electron transport from water to Q_B is fully functional in PS II membranes, of which the 33 kDa protein is totally immobilized.

Identification of crosslinked proteins

For identification of a protein crosslinked with the 33 kDa protein, immunoblotting was carried out with eight antisera raised against the apoproteins of CP47 and CP43, the D1 and D2 proteins, the apoproteins of LHC II and the three extrinsic proteins of 33, 23 and 17 kDa [24]. As stated above, two major crosslinked products labeled A and B appeared on treatment of PS II membranes with EDC (Fig. 1). Band A reacted with both anti-47 and anti-33 but not with any other antisera examined (Fig. 4-I and II, and data not shown). The product band appeared when NaCl-washed PS II membranes depleted of the 23 and 17 kDa proteins had been crosslinked (Fig. 4-II) but not when all the three extrinsic proteins had been removed by urea/NaCl-wash prior to crosslinking (Fig. 4-III). These results indicate that band A is the 33 kDa protein crosslinked with the 47 kDa protein. Band B was

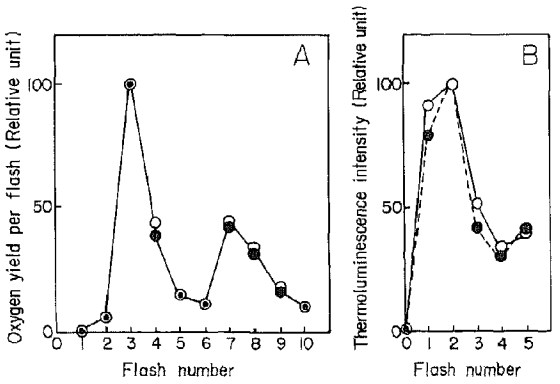

Fig. 3. Oscillation patterns of flash-yield of O_2 (A) and thermoluminescence B band (B). ○, untreated PS II membranes; ●, PS II membranes with about 97% of the 33 kDa protein immobilized with 0.75% EDC.

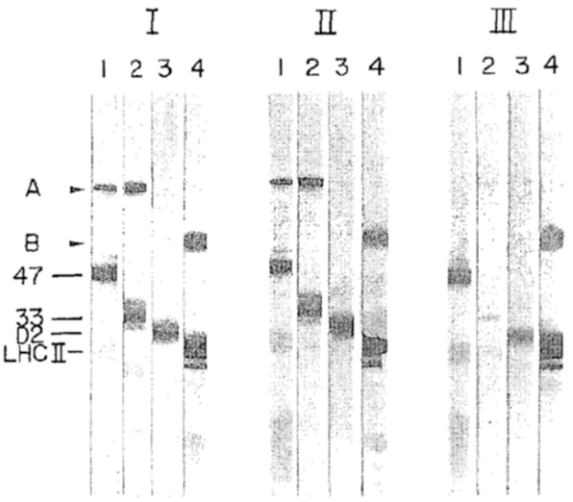

Fig. 4. Immunological identification of proteins crosslinked. I, PS II membranes; II, NaCl-washed PS II membranes; III, urea/NaCl-washed PS II membranes. All the PS II membranes were treated with 0.1% EDC for 10 min. Proteins electrotransfered to nitrocellulose membranes were reacted with the antisera against the 47 kDa protein (lane 1), the 33 kDa protein (lane 2), the D2 protein (lane 3) and LHC II apoproteins (lane 4).

identified as a crosslinked product of the LHC-II apoproteins because the band reacted with anti-LHC-II but none of other antisera (Fig. 4 and data not shown).

The product A has an apparent molecular mass of about 80 kDa, which corresponds to the sum of the molecular masses of the 33 kDa and 47 kDa proteins. This suggests that the 33 kDa protein crosslinks with the 47 kDa protein at a one-to-one ratio. This stoichiometry of crosslinking is supported by the following two observations. Fig. 5 shows that CBB-stained bands of the uncrosslinked 33 kDa and 47 kDa proteins decreased in parallel to each other, as PS II membranes had been treated with increasing concentrations of EDC. This is what would be expected if the two proteins are present in equimolar quantities and covalently linked to each other at a one-to-one ratio.

Parallel disappearances of the 33 kDa and 47 kDa proteins were also demonstrated by immunoblotting with the specific antisera (Fig. 6). In this particular experiment, CBB-stained gel showed that the extrinsic 23 and 17 kDa proteins were also crosslinked at high EDC concentrations. However, note that, besides band A, there was no product band which reacts with either anti-33 or anti-47 at all the crosslinker concentrations examined. The intensity of band A decreased at high concentrations of EDC but this may be ascribed to intensive modification of the proteins with the crosslinker, which would reduce their immunological reactivity. These results altogether indicate that all the 33 kDa protein molecules are immobilized by specifi-

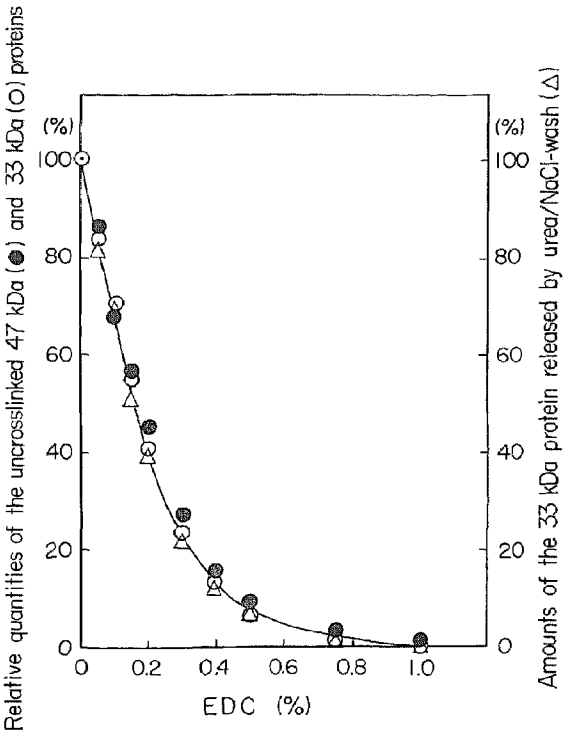

Fig. 5. Relative quantities of the uncrosslinked 47 kDa and 33 kDa proteins in PS II membranes treated with various concentrations of EDC. Crosslinking and gel electrophoresis were carried out as in Fig. 1. ○, ●, relative amounts of the uncrosslinked 33 kDa and 47 kDa proteins determined by measuring peak heights of the protein bands, respectively; △, relative amounts of the uncrosslinked 33 kDa protein estimated by extracting with urea/NaCl treatment as in Fig. 2.

cally crosslinking with the 47 kDa protein molecules a one-to-one stoichiometry.

Stabilization of oxygen evolution

The previous works showed that immobilization of small fraction of the 33 kDa protein with anoth crosslinker, DSP, is accompanied by a parallel increa in the oxygen-evolving activity that survived sever inhibitory treatments [23]. EDC-treatment offers a excellent opportunity to investigate the stabilizing e fect of the protein crosslinking because the 33 kD protein can be 100% immobilized in its fully function state. Fig. 7 shows effects of urea/NaCl-wash on th oxygen-evolving activity of PS II membranes treate with various concentrations of EDC. Extents of th immobilization of the 33 kDa protein were determine by measuring amounts of the 33 kDa protein solub lized by the treatment (curve d). Urea/NaCl treatme of uncrosslinked PS II membranes resulted in mo than 90% inhibition of the oxygen-evolving activi (curve a). As expected, the activity became resistant the treatment as the 33 kDa protein had bee crosslinked. However, even in the membranes with th 33 kDa protein 100% immobilized, the rate of oxyge evolution survived the treatment was only about 40 of that in the unwashed membranes. This can be part ascribed to the depletion of the 23 and 17 kDa protei because the activity was significantly enhanced by adc tion of 5 mM Ca^{2+} (curve b) [27]. The maximu activity thus attained corresponds to about 60% of th original activity. High concentrations of Cl^- and Ca^{2} are known to restore substantial rates of oxygen evol

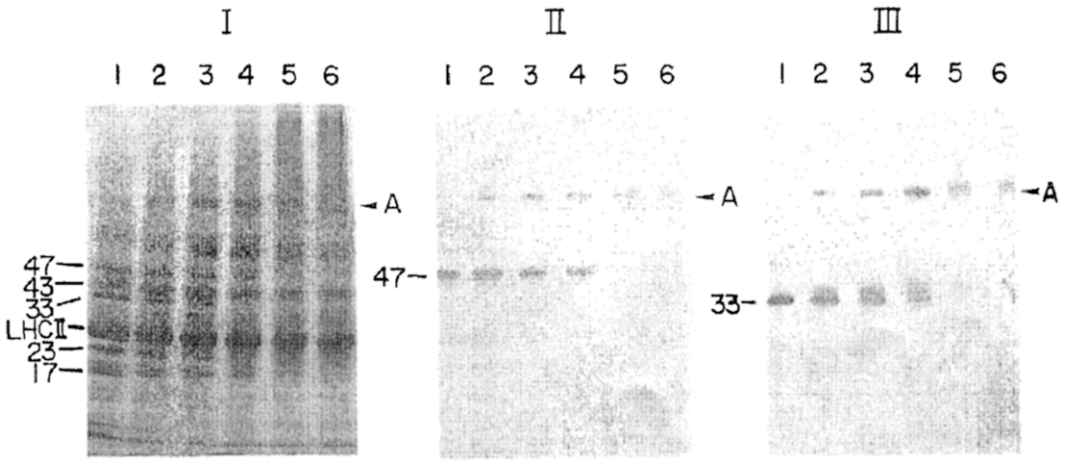

Fig. 6. Immunoblotting of PS II membranes treated with various concentrations of EDC. I, CBB-stained; II, immunoblotted with the antiseru against the 47 kDa protein; III, immunoblotted with the antiserum against the 33 kDa protein. PS II membranes were treated with EDC at 0 (lane 1), 0.05% (lane 2), 0.1% (lane 3), 0.2% (lane 4), 0.4% (lane 5) and 0.6% (lane 6).

tion in PS II membranes depleted of the 33 kDa protein [16,17,19]. Addition of 50 mM $CaCl_2$ further stimulated oxygen evolution in uncrosslinked membranes but not in the membranes with the 33 kDa protein immobilized (curve c). Thus, the immobilization of the 33 kDa protein stabilizes substantially but not completely oxygen evolution against urea/NaCl-wash. Fig. 7 also shows that the stabilization of oxygen evolution is not linearly related to the amount of the 33 kDa protein crosslinked because, with increasing concentration of EDC, the immobilization of the protein proceeded more rapidly than the stabilization of the activity. Thus, at high concentrations of EDC, oxygen evolution was appreciably stabilized without a signficant crosslinking of the 33 kDa protein. This suggests that the activity is also stabilized by crosslinking of a protein(s) other than the 33 kDa protein. Experiments are in progress to examine crosslinking of other proteins at high concentrations of EDC.

Effects of several other inhibitory treatments were examined and the results are summarized in Table I. The crosslinking of the 33 kDa protein stabilized the oxygen-evolving activity against $CaCl_2$- and $MgCl_2$-wash which also liberate the three extrinsic proteins [8]. However, the activities which survived these treatments were even lower than those surviving the urea/NaCl-wash. Thus, the inactivation of oxygen evolution caused by these treatments can not be ascribed solely to extraction of the extrinsic proteins.

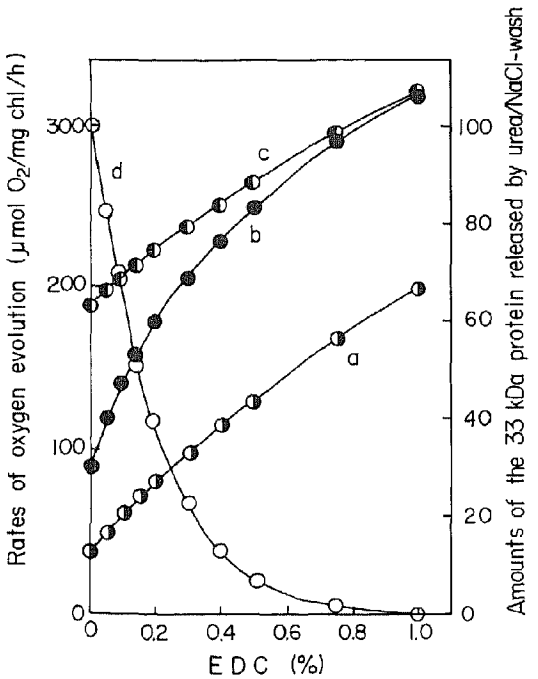

Fig. 7. Effects of urea/NaCl-wash on the oxygen-evolving activity of PS II membranes treated with various concentrations of EDC. PS II membranes treated with indicated concentrations of EDC were washed with 2.6 M urea plus 0.2 M NaCl and rates of oxygen evolution surviving were determined in the absence (a) or presence of 5 mM $CaCl_2$ (b) or 50 mM $CaCl_2$ (c). The rate of oxygen evolution in unwashed membranes was 510 μmol/mg Chl per h. The amounts of the 33 kDa protein solubilized by urea/NaCl-wash (d) were determined as in Fig. 2.

TABLE I

Effects of various treatments on oxygen-evolving activity of PS II membranes crosslinked with 0.5% EDC

Oxygen evolution was measured in the presence of 5 mM Ca^{2+}.

Treatments	Oxygen evolution (μmol/mg Chl per h)	
	uncross-linked	cross-linked
Non-treatment	524	520
2.6 M urea plus 0.2 M NaCl	85	302
1 M $CaCl_2$	50	210
1 M $MgCl_2$	60	202
0.8 M Tris	0	0
2 mM NH_2OH	0	0
Alkaline (pH 9.0)	0	20

Tris treatment resulted in a total inactivation of oxygen evolution of PS II membranes crosslinked with EDC. This is consistent with the previous observation that Tris-wash inactivates oxygen evolution by extracting Mn, irrespective of whether the 33 kDa protein is crosslinked or not [23]. NH_2OH-wash, which is known to extract Mn while leaving the extrinsic proteins bound to membranes [6], completely inactivated PS II membranes with the 33 kDa protein crosslinked.

The previous work showed that crosslinking of up to 20% of the 33 kDa protein with DSP was accompanied by corresponding small increases in the amount of Mn that remained unextracted by and the rate of oxygen evolution survived the treatment of PS II membranes at pH 9.0 for 30 min [23]. The observation led to a conclusion that the 33 kDa protein has a protecting effect on the Mn cluster at pH 9.0. It was therefore unexpected that the oxygen-evolving activity of PS II membranes, of which the 33 kDa protein was 100% immobilized, was almost completely inhibited by pH 9.0 treatment (Table I). The finding prompted us to reinvestigation of effects of EDC and DSP on the activity of PS II membranes exposed to pH 9.0. The alkaline pH-treatment strongly inhibited oxygen evolution of preparations crosslinked with the two crosslinkers. However, the activitiy which survived pH 9.0 treatment was always higher in the membranes, of which 10 to 20% of the 33 kDa protein had been crosslinked with DSP, than in preparations, of which nearly 100% of the protein had been immobilized with EDC (data not shown).

EDC preferentially reacts with carboxyl groups [39],

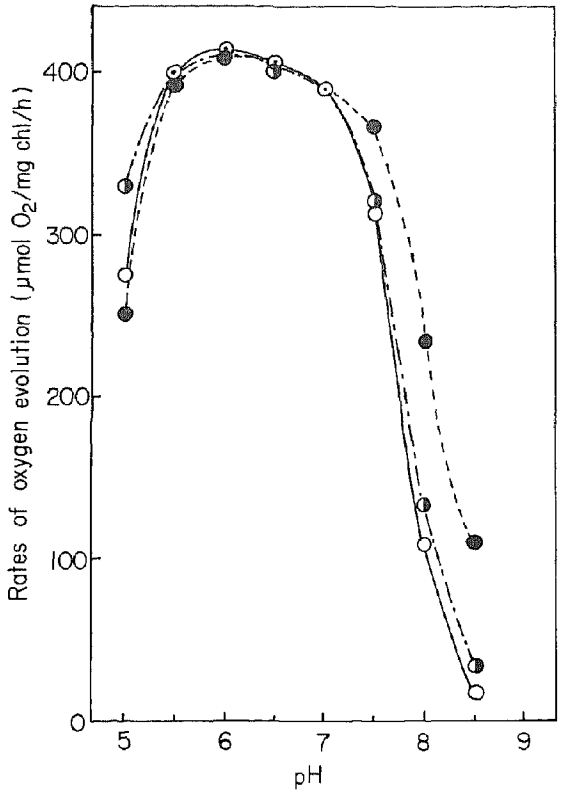

Fig. 8. pH dependence of oxygen evolution of PS II membranes untreated (○) and treated with 0.1% DSP (●) or 0.1% EDC (◑). Buffers used were 50 mM Mes-NaOH (pH 5.0–6.5) and 50 mM Hepes-NaOH (pH 7.0–8.5).

whereas DSP specifically blocks amino groups [40]. Thus, the two reagents will differently affect the electrostatic properties of constituent proteins of the oxygen-evolving complexes. Fig. 8 shows that the rate of oxygen evolution of untreated PS II membranes was maximal between pH 5.5 and 7.0 and sharply decreased at pH levels above 7.5. Oxygen evolution at pH 9.0 could not determined, because phenyl-*p*-benzoquinone was unstable at the alkaline pH. EDC treatment had no significant effect on the pH-dependence of the activity. However, when membranes had been crosslinked with DSP, inactivation of oxygen evolution was slightly but significantly suppressed at higher pH. The results suggest that DSP increases the stability of oxygen evolution at alkaline pH levels by reacting with an amino group(s) or by crosslinking proteins other than the 33 kDa and 47 kDa proteins.

Discussion

Crosslinking between the 33 kDa and 47 kDa proteins has been reported by Enami et al. [21,23,24,29] and Bricker et al. [22]. In these earlier experiments, only a small fraction (5–20%) of the 33 kDa protein was crosslinked because intensive treatments with crosslinking reagents caused inhibition of oxygen evolution [23] or intercomplex crosslinking [22]. The present work demonstrates for the first time that all the 33 kDa protein can be crosslinked without affecting oxygen evolution or the S-state transition.

EDC is a zero-length crosslinker which catalyses the formation of peptide bond between carboxyl and amino groups [39]. Thus, the result indicates that the extrinsic 33 kDa protein attaches directly to the intrinsic 47 kDa protein through an electrostatic interaction. This is not necessarily contradictory to previous works which showed that the 33 kDa protein binds to the oxygen-evolving complexes through hydrogen bonding or hydrophobic interaction [41–43], but emphasizes the involvement of various weak interactions in the binding of the 33 kDa protein to the 47 kDa and other subunit proteins of PS II complexes. Binding of the 33 kDa protein to the D1 and D2 proteins [44], the extrinsic 23 kDa protein [24,45] and the 43 kDa chlorophyll-carrying protein [46] has been reported.

Although the consensus exists that the three extrinsic proteins of 33, 23 and 17 kDa proteins are present in equimolar amounts in PS II preparations [12–14], there is still a controversy as to the copy number of the proteins associated with the oxygen-evolving complex. Analysis of CBB-stained bands of PS II preparation led to the stoichiometry of one copy of each of the three extrinsic proteins per PS II reaction center [12]. In contrast, crosslinking experiments suggested that two copies of each of the extrinsic proteins are present per PS II [15]. The present work indicates that the 33 kDa protein is crosslinked to the 47 kDa protein at a one-to-one ratio. With increasing concentrations of the crosslinker, the uncrosslinked 33 kDa and 47 kDa proteins decreased in parallel, yielding only a single crosslinked product with an apparent molecular mass of 80 kDa. These results indicate that the 33 kDa and 47 kDa proteins are present in equimolar amounts in PS II membranes and hence support the stoichiometry of one copy each of the extrinsic proteins per PS II reaction center.

The results obtained here differ from the previous observations that EDC yielded two crosslinked products of 76 and 65 kDa which reacted with anti-33 and anti-47 and, with increasing concentration of EDC, there was a gradual loss of the uncrosslinked 47 kDa protein which was independent of either decrease in the amount of the uncrosslinked 33 kDa protein or the formation of the product bands [22]. At present, the reason for the discrepancies is not known.

Inactivation of oxygen evolution by urea/NaCl-, $CaCl_2$- and $MgCl_2$-wash is accompanied by liberation of the three extrinsic proteins and the lost activity is

substantially restored by reconstitution of the 33 kDa protein in the presence of Ca^{2+} and Cl^- [17]. Thus, these treatments are generally considered to suppress oxygen evolution through solubilization of the extrinsic proteins, in particular, the 33 kDa protein [8,16]. As expected, immobilization of the 33 kDa protein considerably stabilized the oxygen-evolving activity against these treatments. This provides additional evidence for the important role that the 33 kDa protein plays in the oxygen-evolving reaction. However, the total immobilization of the 33 kDa protein failed to give rise complete protection of oxygen evolution. This could be, at least partially, ascribed to the removal of the 23 kDa and 17 kDa proteins, because the activity resistant to the treatments was increased on addition of Ca^{2+}. However, even when determined in the presence of sufficiently high concentrations of Ca^{2+} and Cl^-, the activity survived these treatments was still low, amounting only to 40–60% of the original activity. These results suggest that, besides extraction of the extrinsic proteins, these treatments exert an additional inhibitory effect on the oxygen evolution. This cannot be related to extraction of Mn because all the these treatments are known to leave Mn bound to its functioning site [8,16]. Thus, immobilization of the extrinsic protein(s) is expected to give new insight into the inhibition mechanisms of the treatments.

Oxygen evolution became less sensitive to alkaline pH treatment when membranes had been crosslinked with DSP [23]. The present work shows that the effect of DSP cannot be related to crosslinking of the 33 kDa and 47 kDa proteins because the total immobilization of the 33 kDa protein with EDC had no stabilizing effect. DSP, but not EDC, was found to affect the pH-dependence of oxygen evolution in the alkaline pH region. Whereas the activity half decreased at about pH 7.8 in both untreated and EDC-treated membranes, the pH for the half-maximum activity was 8.1 after DSP treatment (Fig. 8). DSP reacts highly specifically with amino groups [40]. An implication is therefore that DSP affects the pH-dependence or pH-sensitivity of oxygen evolution by reacting with amino groups of the functional subunits of PS II complex. At any event, the results obtained here caution that effects of crosslinking treatments cannot always be related to crosslinking of proteins.

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