Unassembled subunits of the photosynthetic oxygen-evolving complex present in the thylakoid lumen are long-lived and assembly-competent

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Abstract Physiologically healthy pea chloroplasts contain unassembled, soluble subunits of the oxygen-evolving complex in the thylakoid lumen. We report that the lifetimes of two of these subunits, both on and off the membrane, exceed 8 h in vitro. We also demonstrate that each of the subunits present in the thylakoid lumen is competent for assembly into the membrane-bound complex. These data are consistent with the postulate that the soluble lumen-resident subunits play a role in photosystem II homeostasis. We also demonstrate that the reconstitution of the 33 kDa subunit is inhibited by extremely low concentrations of Triton X-100, suggesting that hydrophobic interactions are involved in the binding of this subunit to the photosystem II reaction center.

Key words: Chloroplast; Photosystem II; Oxygen evolution

1. Introduction

Many experiments have shown that, as a general rule, chloroplast- and nuclear-encoded chloroplast proteins are either assembled immediately upon import from the cytoplasm or they are rapidly degraded. Examples of polypeptides that behave in this fashion include apoplastocyanin, the small subunit of ribulose bisphosphate carboxylase/oxygenase, light harvesting chlorophyll a/b binding protein, D1, CP43 and subunits of the chloroplast coupling factor [1–6]. It has been suggested that this mechanism may help to ensure the proper balance between subunits derived from the different genomes [1,6,7].

The three extrinsic nuclear-encoded subunits of the photosynthetic oxygen-evolving complex (OEC[2]), termed OE33, OE23 and OE17 for their respective molecular weights, apparently do not conform to this general rule. A number of studies have shown that relatively normal amounts of OEC subunits often accumulate in mutants which do not assemble the PS II reaction center due to defects in chloroplast-encoded core subunits [8–11]. Similarly, etiolated plastids in barley which do not contain a functional PS II core were observed to accumulate low but stable levels of OEC subunits [12].

In concert with these observations, Ettinger and Theg [13] discovered relatively large pools of soluble OEC subunits present in the thylakoid lumen of physiologically normal chloroplasts from pea and spinach. Subsequently, Palomares et al. [14] identified OEC subunits in a soluble fraction derived from tobacco leaves. Further, in the photoinhibited chloroplasts, the PS II-assembled OEC subunits were observed to be released into the thylakoid lumen as PS II complexes were disassembled [15,16]. More recently, van Wijk et al. [17] showed that import of newly synthesized nuclear-encoded proteins (including OEC subunits) was not required for membrane integration of newly synthesized D1 protein and its subsequent reassembly into new PS II complexes.

These data imply that the pool of unassembled OEC subunits present in physiologically healthy chloroplasts is involved in normal OEC homeostasis. If this is true, then the subunits residing in the soluble lumen pool must have relatively long half-lives, and must be fully competent for assembly as binding sites become available. The experiments reported in this communication confirm these predictions and provide new insight into the mechanism of assembly and maintenance of PS II.

2. Materials and methods

2.1. Plant material, isolation of chloroplasts and protein import reactions

Pea seedlings (*Pisum sativum* cv. Progress #9) were grown in moist vermiculite for 11 days in a 14 h light at 21°C/10 h dark at 19°C cycle. Intact chloroplasts were isolated on a linear Percoll gradient as described by Ettinger and Theg [13] and resuspended at ≥1 mg chl/ml in 333 mM sorbitol and 50 mM K-Tricine at pH 8.0 (import buffer). Chloroplast import of in vitro-synthesized OEC subunits was performed in dim white light (150 μE m^{-2} s^{-1}) essentially as described by Cline et al. [18].

2.2. Subfractionation of chloroplasts

For subfractionation of intact chloroplasts, the isolated chloroplasts were resuspended with a lysis buffer (10 mM Mes-NaOH pH 6.5, 5 mM $MgCl_2$) to break the envelope membranes. They were vortexed and incubated for 5 min on ice, and then centrifuged at 7500×*g* for 5 min. The pellet was washed with lysis buffer again and was subsequently resuspended with thylakoid buffer (40 mM Mes-NaOH at pH 6.5, 10 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose). For further fractionation, the thylakoids were incubated with 0.1% Triton X-100 (diluted in thylakoid buffer) for 3 min on ice and centrifuged at 144 000×*g* for 15 min. The pellet and supernatant were recovered as the membrane and the lumen fractions, respectively. Alternatively, thylakoids were sonicated by two 15 s bursts of a probe sonicator interrupted by a 15 s cool-down period on ice, followed by centrifugation as above. During these fractionation procedures, the concentration of chlorophyll was maintained at 0.5 mg/ml.

2.3. Preparation of PS II particles and OEC-depleted PS II

PS II complexes were prepared from pea leaves essentially as described by Ikeuchi and Inoue [19] using 4% Triton X-100. The PS II membranes were resuspended with thylakoid buffer (described above) containing 30% (w/v) glycerol and kept at −80°C until use.

PS II membranes depleted of both OE23 and OE17 were prepared by washing the PS II particles with MNS buffer (40 mM Mes-NaOH

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Abbreviations: Chl, chlorophyll; OEC, oxygen-evolving complex; OE33, OE23 and OE17, 33, 23 and 17 kDa subunits of the OEC; PS II, photosystem II

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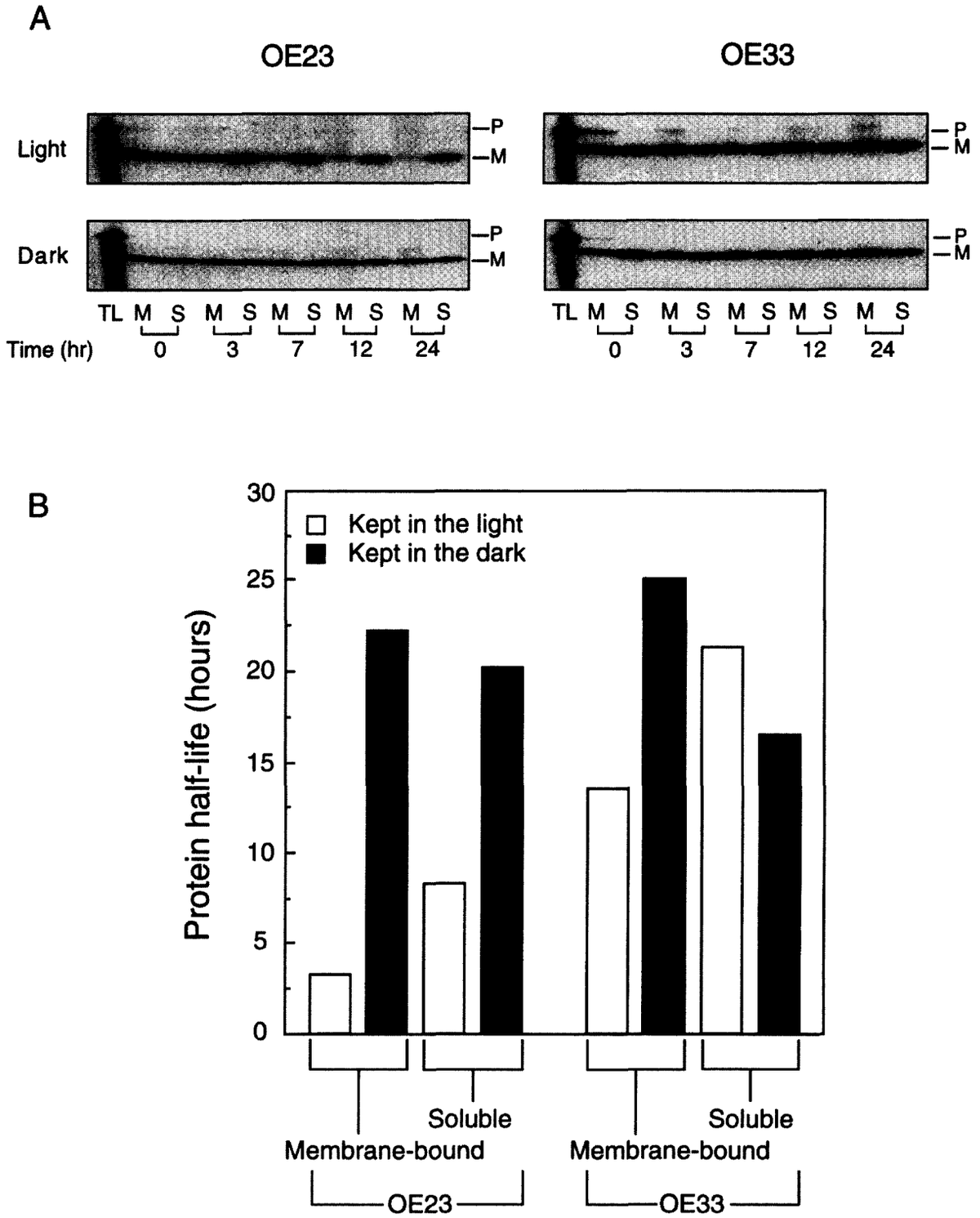

Fig. 1. Half-lives of newly imported OE33 and OE23 on the membrane and free in the lumen. Protein import reactions were carried out using intact chloroplasts in the light for 40 min. After re-purification of the plastids by centrifugation through 40% Percoll, they were resuspended in import reaction buffer and incubated at room temperature in the light or in the dark. At the times indicated, samples were withdrawn and fractionated into the thylakoid membrane and lumen components. They were subjected to SDS-PAGE and fluorography, and the mature proteins were quantitated by densitometry.

at pH 6.5, 2 M NaCl and 0.4 M sucrose) [20,21]; membranes depleted of all three OEC subunits were prepared by a wash with MNSC buffer (40 mM Mes-NaOH pH 6.5, 10 mM NaCl, 0.4 M sucrose, 1. 2 M $CaCl_2$) [22].

2.4. In vitro binding assay of the OEC subunits into the PS II complexes

The OEC subunits dissociated from the PS II particles by washing in MNS or MNSC buffer, or collected with the lumen prepared from

isolated chloroplasts, were concentrated about 100-fold and simultaneously desalted with a Centricon #10 centrifugal concentrator (10 000 kDa cut-off, Amicon). To investigate the assembly competence of the unassembled soluble OEC subunits in the lumen, 135 μg of the concentrated lumen was mixed with MNS- or MNSC-washed PS II particles corresponding to 25 μg chl. As a control, 10 μg (for OE33) or 35 μg (OE23 and OE17) of the concentrated PS II-derived OEC subunits was added to the MNS-washed or MNSC-washed PS II particles, respectively. After incubation on ice for 30 min in the dark, the reaction mixture was centrifuged at 40 000×*g* for 5 min, and pelleted material was washed once and resuspended in thylakoid buffer. The resulting reconstituted PS II particles were subjected to SDS-urea-PAGE and the proteins were visualized by Coomassie staining.

2.5. Purification of the unassembled soluble OE33 from the lumen

Purification of the soluble OE33 from the concentrated lumen was carried out by FPLC cation exchange chromatography (Pharmacia). The concentrated lumen was diluted approximately 20-fold in 20 mM Mes-NaOH at pH 6.0 and absorbed to Hitrap SP column equilibrated with the same buffer flowing at 2 ml/min. The absorbed material was eluted with 20 ml of this buffer containing a 0–200 mM NaCl gradient; OE33 eluted at approximately 30 mM NaCl. The purified OE33 was concentrated and desalted using a Centricon #3 centrifugal concentrator subsequent to use in reconstitution assays.

3. Results

The postulate that the free OEC subunits present in the thylakoid lumen play a role in PS II homeostasis, providing perhaps a ready supply of subunits to be utilized in repair after D1 turnover, minimally requires that they have relatively long half-lives. To test this prediction we measured the turnover times of OE33 and OE23 that had been newly imported into intact chloroplasts and correctly localized to the thylakoid lumen. Salt-washing experiments suggest that newly imported subunits binding to the membrane from the lumen side are assembled into OECs in a physiological manner (Hashimoto et al., manuscript in preparation). After the appropriate import reactions were performed and terminated, the samples were placed either in the light (150 μE m^{-2} s^{-1}) or in darkness at room temperature, and aliquots were withdrawn at the indicated times. The aliquots were subsequently fractionated into membrane and soluble fractions, and the relative amounts of imported protein residing on the membrane or in the lumen pool were assessed by fluorography. As can be seen in Fig. 1, the subunit half-lives were generally shorter in the light than in the dark, and this trend was exacerbated by association with the membrane. Although the light intensity for long-term illumination was relatively low, it would not be surprising if long-term illumination, especially in the absence of electron acceptors, caused degeneration of the isolated plastids. More remarkable is the observation in Fig. 1 of the long half-lives of the OEC subunits in samples kept in the dark. As pointed out by many authors (cf. [7]), subunits of multimeric protein complexes are generally unstable if they are not quickly incorporated into their respective complexes. That the OEC subunits are exceptions to this rule is confirmed by the fact that the respective half-lives of OE23 and OE33 in the lumen pool in the dark exceeded 15 h (Fig. 1, dark bars). It is apparent that the residence time of OEC subunits in the lumen pool, even in the light, would not be a hindrance to their playing a role in PS II homeostasis.

A second requirement of a role of free lumen-resident OEC subunits in PS II homeostasis is that the subunits remain competent for assembly into the enzyme complex under appropriate conditions. To test this we prepared PS II particles stripped of either OE23 and OE17 by washing in 2 M NaCl, or of all three extrinsic subunits by washing with 1.2 M $CaCl_2$ (see Section 2). These extrinsic subunit-depleted membranes were then incubated with OEC subunits purified from active PS II particles, or present in the lumen pool (Fig. 2). Following incubation, the PS II membranes were recovered by centrifugation and assayed for the rebinding of the subunits. Lanes 5 and 8 document the well-known results of such a reconstitution assay when the subunits are derived from active oxygen-evolving PS II particles. When a preparation of lumen was incubated with PS II particles stripped by NaCl washing, OE17 and OE23 rebound to levels approaching their original values (compare lanes 1, 5 and 6), thereby proving their inherent assembly competence. A different result was obtained when PS II particles stripped of OE33 by $CaCl_2$ were incubated with a lumen fraction containing soluble OE33. We consistently observed that, although efficient reconstitution occurred when OE33 was derived from active PS II particles, OE33 present in the lumen fraction did not bind to stripped PS II particles (compare lanes 8 and 9 in Fig. 2).

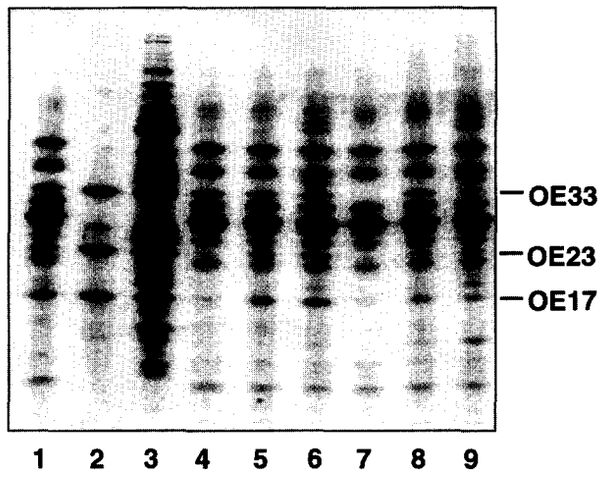

Fig. 2. OE23 and OE17, but not OE33, released from the lumen by Triton X-100 are competent for assembly onto subunit-depleted PS II membranes. In vitro binding assays were carried out using lumen fractions isolated after treatment of thylakoids with 0.1% Triton X-100 as described in Section 2. Lane 1, PS II complexes prepared from pea thylakoids. Lane 2, concentrated and desalted OEC subunits dissociated from the PS II particles with MNSC. Lane 3, concentrated lumen fraction. Lane 4, MNS-washed PS II particle. Lane 5, dissociated OEC subunits (lane 2) were incubated with MNS-washed PS II particles (lane 4). Lane 6, concentrated lumen (lane 3) was incubated with MNS-washed PS II particles (lane 4). Lane 7, MNSC-washed PS II particles. Lanes 8 and 9, as in lanes 5 and 6, respectively, except MNSC-washed PS II particles were used.

The surprising inability of OE33 in the lumen to bind PS II membranes was traced to the procedure we utilized to isolate the lumen. The experiment shown in Fig. 3A demonstrates that when assembly-incompetent, lumen-resident OE33 was purified to homogeneity from the lumen fraction by ion exchange chromatography (lane 3) and incubated with MNSC-washed PS II membranes (lane 2), efficient reconstitution was observed (lane 5). Conversely, the addition of the lumen fraction (lane 4) to a similar reconstitution reaction containing assembly-competent OE33 purified from active PS II particles resulted in the inhibition of the latter's binding to stripped PS

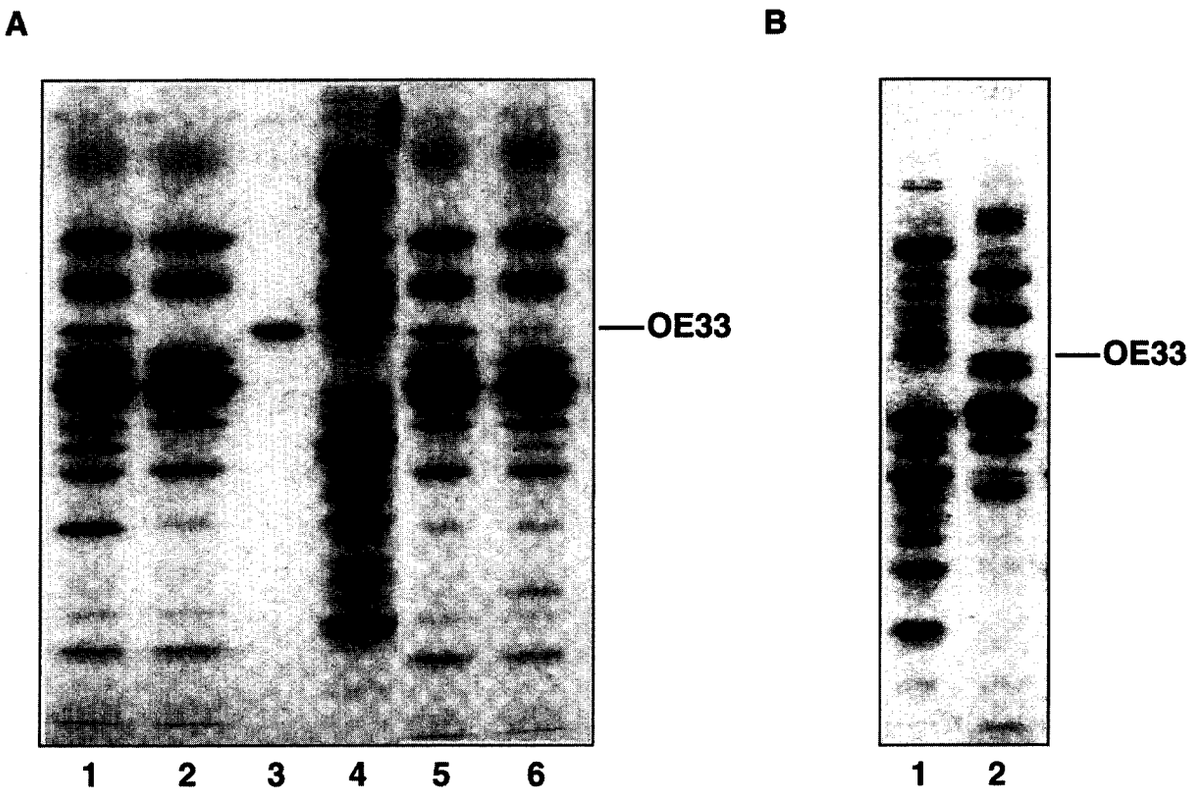

Fig. 3. OE33 reconstitution reactions. A: Reconstitution reactions using OE33 purified from the lumen in the presence and absence of the lumen fraction. Lane 1, PS II particle. Lane 2, MNSC-washed PS II particle. Lane 3, unassembled, lumen-resident OE33 purified from the thylakoid lumen as described in Section 2. Lane 4, detergent-released lumen fraction. Lane 5, reconstitution of MNSC-washed PS II (lane 2) with purified OE33 (lane 3). Lane 6, as in lane 5, but lumen fraction (lane 4) added to the reconstitution reaction. B: Reconstitution reactions performed with lumen released by sonication. Lane 1, sonication-released lumen fraction. Lane 2, reconstitution of MNSC-washed PS II particle (lane 2 in panel A) with sonication-released OEC subunits (lane 1).

II particles (lane 6). These experiments suggested that a component of the lumen fraction, very likely residual Triton X-100 (see below), was responsible for the observed inhibition of the OE33 assembly reaction. Fig. 3B demonstrates that when the lumen fraction was prepared by sonication of the membranes rather than detergent lysis (lane 1), lumen-resident OE33 appeared fully competent for assembly (lane 2).

The experiments shown in Figs. 2 and 3 collectively indicate that the OEC subunits that are present in the soluble pool within the lumen are competent for assembly into newly forming or reforming OEC complexes.

The experiments described above strongly suggested that the inhibitory effect of the lumen on the binding of OE33 to PS II particles was due to the residual Triton X-100 added to thylakoids to prepare the lumen fraction. This was unexpected. On the one hand, the detergent was added to a maximum concentration of 0.1% to lyse the thylakoids and then diluted considerably during concentration of the lumen proteins. On the other, Triton X-100 is routinely used by us and others [19] at 4% to prepare oxygen-evolving, OE33-containing PS II membranes. We therefore further investigated this detergent-induced inhibition of the OE33 reconstitution reaction in the experiments shown in Fig. 4. Fig. 4A demonstrates that the concentration of Triton X-100 remaining in our lumen preparations was insufficient to cause thylakoid lysis, placing it below 0.02% [13]. In concert with this, titrations of the effect of Triton X-100 on the OE33 reconstitution reaction resulted in inhibition at concentrations as low as 0.005–0.01% (Fig. 4B). Since these inhibitory concentrations are below the critical micelle concentration for Triton X-100 [23], we can conclude from this experiment that the detergent effect on OE33 binding was not due to preferential sequestration of OE33 into micelles.

4. Discussion

We have shown previously that the subunits of the oxygen-evolving enzyme complex are present in substantial quantities (i.e. typically 20–50% of the total [13]; Hashimoto and Theg, unpublished) in soluble pools in the thylakoid lumen of physiologically healthy chloroplasts. In this communication we address three previously undocumented points regarding these soluble OEC subunits. First, we show that at least the 33 and 23 kDa subunits in the pool are rather stable, both in the light and in the dark. We should point out, however, that we attach more significance to the data obtained with the samples held in the dark because of the potential for nonspecific damage incurred by illuminating isolated plastids for long periods of time in the absence of electron acceptors. Second, we demonstrate that each of the three subunits in the lumen is fully competent for assembly onto subunit-depleted PS II membranes. Finally, while having no effect on the binding of OE23 and OE17 to stripped PS II membranes, we found

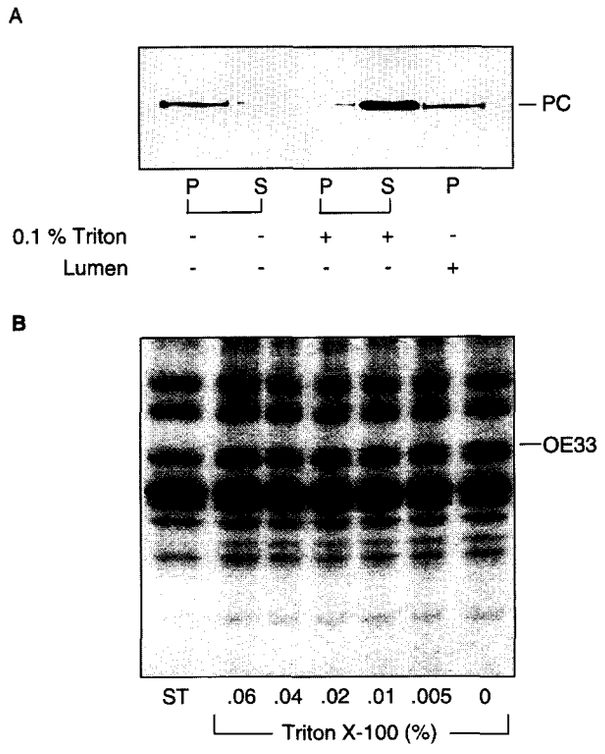

Fig. 4. Effects of residual Triton X-100 on thylakoid integrity and OE33 reconstitution. A: Residual Triton X-100 in the detergent-released lumen fraction does not cause lysis of intact thylakoids. When present, Triton X-100 was added to intact thylakoids, which were subsequently centrifuged to separate soluble (lumen) and membrane thylakoid components. Fractions recovered were Western blotted with anti-plastocyanin, used here as a marker of soluble lumen contents (cf. [13]). In the final lane, concentrated and desalted lumen prepared by similar treatment of thylakoids with 0.1% Triton (see Section 2) was added to intact thylakoids, which were then subjected to centrifugation; only the pellet is shown because the supernatant was heavily contaminated by plastocyanin brought into the experiment through the added lumen preparation. B: Titration of the inhibitory effect of Triton X-100 on OE33 reconstitution of MNSC-washed PS II particles. Reconstitution of MNSC-washed PS II particles with OE33 was performed as in lane 8 of Fig. 2 in the presence of the indicated concentrations of Triton X-100.

that Triton X-100, at remarkably low concentrations, inhibits the binding of OE33 to PS II.

As mentioned in Section 1, protein turnover appears to be one of the mechanisms utilized by cells to maintain proteins with their proper subunit stoichiometry [7]. This is true in many cellular compartments, including chloroplasts, where the appearance of unassembled subunits triggers their breakdown, presumably by specific proteases. Proteases that specifically degrade OEC subunits from spinach have been investigated by Kuwabara and Suzuki [24–26]. Distinct proteases that cleaved at specific locations in OE17 and OE23 were partially purified from PS II-containing membranes. These proteases apparently do not degrade fully folded and assembled subunits, requiring instead some conformational changes before recognizing their substrates. In a related work, Roffey and Theg [27] showed that C-terminally truncated OE23 subunits were degraded immediately upon import into the thylakoid lumen. These truncated subunits were shown to possess non-native conformations, which presumably signalled them for degradation, perhaps by the same proteases as studied by Kuwabara and Suzuki. To our knowledge, proteases acting on OE33 have not been described, although we have no reason to suspect they do not exist.

It is obvious that chloroplasts have a clear propensity for degrading unassembled subunits, and that proteases which hydrolyze OEC subunits under certain conditions are known to be present in the thylakoid lumen, one has to ask why soluble, assembly-competent OEC subunits are allowed to defeat these proteases and reside in pools in the lumen. The answer must lie in the unique requirements for PS II homeostasis. Given the high turnover rate of the D1 reaction center core protein, coupled with the deleterious effects on chloroplast integrity of long-lived oxidants at the reaction center (reviewed in [28]), it is probable that activation of PS II reaction centers without activation of attached OECs is extremely harmful. In this context, the OEC subunits can be seen as contributing a steric shield around the PS II reaction center, as well as a functional oxidant trap for P_{680}^{+}. The presence and maintenance of soluble, assembly-competent OEC subunits in the thylakoid lumen would then provide a ready source of material from which newly formed OECs may be assembled as soon as PS II is reassembled.

It is noteworthy that the assembly of OE33 onto PS II membranes with open OE33 binding sites is inhibited by extremely low concentrations of Triton X-100. The fact that this subunit can be washed from membranes by high levels of $CaCl_2$, or by NaCl in conjunction with urea, suggests that it is bound electrostatically to the PS II reaction center. Our new observation that OE33 reconstitution is potently inhibited by detergent suggests that hydrophobic interactions are also critical in the binding of this subunit. We can, for instance, rule out the possibility that Triton X-100 forces OE33 into an assembly-incompetent conformation by the fact that this subunit can be recovered from detergent-treated PS II particles in a reconstitutable form. We postulate instead that one or more hydrophobic patches may be exposed on the surface of native OE33, and that the binding pocket for this subunit on PS II reaction centers may contain complementary hydrophobic faces. The binding of detergent to the surface of the subunit would then prevent favorable hydrophobic interactions in the OE33 binding pocket, thereby inhibiting formation of further stabilizing salt bridges. In this light, the lack of an inhibitory effect of detergent on OE23 and OE17 reconstitution would suggest that the binding of these subunits is purely ionic. This may also explain why these smaller subunits dissociate from the reaction center under milder denaturing conditions than those required for OE33 dissociation.

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References

[1] Schmidt, G.W. and Mishkind, M.L. (1983) Proc. Natl. Acad. Sci. USA 80, 2632–2636.
[2] Bennett, J. (1981) Eur. J. Biochem. 118, 61–70.
[3] Mullet, J.E., Klein, P.G. and Klein, R.R. (1990) Proc. Natl. Acad. Sci. USA 87, 4038–4042.
[4] Merchant, S. and Selman, B.R. (1984) Plant Physiol. 75, 781–787.
[5] Li, H.H. and Merchant, S. (1995) J. Biol. Chem. 270, 23504–23510.

[6] Biekmann, S. and Feierabend, J. (1985) Eur. J. Biochem. 152, 529–535.
[7] Luzikov, V.N. (1986) FEBS Lett. 200, 259–264.
[8] Rochaix, J.-D. and Erickson, J. (1988) Trends Biochem. Sci. 13, 56–59.
[9] Mayfield, S.P., Schirmer-Rahire, M., Frank, G., Zuber, H. and Rochaix, J.-D. (1989) Plant Mol. Biol. 12, 683–693.
[10] De Vitry, C., Olive, J., Drapier, D., Recouvreur, M. and Wollman, F.-A. (1989) J. Cell Biol. 109, 991–1006.
[11] Nilsson, F., Andersson, B. and Jansson, C. (1990) Plant Mol. Biol. 14, 1051–1054.
[12] Hashimoto, A., Akasaka, T. and Yamamoto, Y. (1993) Biochim. Biophys. Acta 1183, 397–407.
[13] Ettinger, W.F. and Theg, S.M. (1991) J. Cell Biol. 115, 321–328.
[14] Palomares, R., Herrmann, R.G. and Oelmueller, R. (1993) Eur. J. Biochem. 217, 345–352.
[15] Hundal, T., Aro, E.-M., Carlberg, I. and Andersson, B. (1990) FEBS Lett. 267, 203–206.
[16] Eisenberg-Domovich, Y., Oelmuller, R., Herrmann, R.G. and Ohad, I. (1995) J. Biol. Chem. 270, 30181–30186.
[17] Van Wijk, K.J., Bingsmark, S., Aro, E.M. and Andersson, B. (1995) J. Biol. Chem. 270, 25685–25695.
[18] Cline, K., Ettinger, W.F. and Theg, S.M. (1992) J. Biol. Chem. 267, 2688–2696.
[19] Ikeuchi, M. and Inoue, Y. (1986) Arch. Biochem. Biophys. 247, 97–107.
[20] Akerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10.
[21] Kuwabara, T. and Murata, N. (1983) Plant Cell Physiol. 24, 741–747.
[22] Ono, T.-A. and Inoye, Y. (1983) FEBS Lett. 164, 255–260.
[23] Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79.
[24] Kuwabara, T. (1992) FEBS Lett. 300, 127–130.
[25] Kuwabara, T. and Suzuki, K. (1994) Plant Cell Physiol. 35, 665–675.
[26] Kuwabara, T. and Suzuki, K. (1995) Plant Cell Physiol. 36, 495–504.
[27] Roffey, R. and Theg, S. (1996) Plant Physiol. (in press).
[28] Aro, E.-M., Virgin, I. and Andersson, B. (1993) Biochim. Biophys. Acta Bio-Energetics 1143, 113–134.