Title

Physiologically active chloroplasts contain pools of unassembled extrinsic proteins of the photosynthetic oxygen-evolving enzyme complex in the thylakoid lumen.

Journal

Journal of Cell Biology, 115(2)

ISSN

0021-9525

Authors

Ettinger, WF
Theg, SM

Publication Date

1991-10-15

DOI

10.1083/jcb.115.2.321

Peer reviewed

Physiologically Active Chloroplasts Contain Pools of Unassembled Extrinsic Proteins of the Photosynthetic Oxygen-evolving Enzyme Complex in the Thylakoid Lumen

William F. Ettinger and Steven M. Theg

Department of Botany, University of California, Davis, California 95616

Abstract. The oxygen-evolving complex (OEC) of photosystem II (PS II) consists of at least three extrinsic membrane-associated protein subunits, OE33, OE23, and OE17, with associated Mn^{2+}, Ca^{2+}, and Cl^- ions. These subunits are bound to the lumen side of PS II core proteins embedded in the thylakoid membrane. Our experiments reveal that a significant fraction of each subunit is normally present in unassembled pools within the thylakoid lumen. This conclusion was supported by immunological detection of free subunits after freshly isolated pea thylakoids were fractionated with low levels of Triton X-100. Plastocyanin, a soluble lumen protein, was completely released from the lumen by 0.04% Triton X-100. This gentle detergent treatment also caused the release from the thylakoids of between 10 and 20%, 40 and 60%, and 15 and 50% of OE33, OE23, and OE17, respectively. Measurements of the rates of oxygen evolution from Triton-treated thylakoids, both in the presence and absence of Ca^{2+}, and before and after incubation with hydroquinone, demonstrated that the OEC was not dissociated by the detergent treatment. Thylakoids isolated from spinach released similar amounts of extrinsic proteins after Triton treatment. These data demonstrate that physiologically active chloroplasts contain significant pools of unassembled extrinsic OEC polypeptide subunits free in the lumen of the thylakoids.

PHOTOSYSTEM II (PS II)[1] is a functional unit in chloroplasts containing light-harvesting and electron transporting proteins, and is located primarily in the appressed regions of chloroplast thylakoid membranes. The PS II reaction center core consists of the integral membrane proteins CP47, CP43, D1, D2, and cytochrome b-559, each of which is encoded in the chloroplast genome (reviewed in Ghanotakis and Yocum, 1990). The oxygen-evolving complex (OEC) of PS II is made up of at least three nuclear-encoded polypeptides, named according to their relative molecular weights, OE33, OE23, and OE17. The polypeptides have no known enzymatic activity by themselves, and their individual contributions to oxygen evolution are not completely understood (for example see Burnap and Sherman, 1991). The OEC is tightly bound to the lumen face of the PS II reaction center core (Miyao and Murata, 1989). The OEC polypeptides can be dissociated from the PS II core by a number of inhibitory treatments, including washing with alkaline Tris, 2 M NaCl, 1 M $CaCl_2$, hydroxylamine, and chaeotropic agents, or by physical means such as heat treatment (reviewed in Babcock, 1987; Ghanotakis and Yocum, 1990). In PS II sub-chloroplast particles, optimal reactivation of the OEC after such treatments requires the addition of the individual subunits (reviewed in Ghanotakis and Yocum, 1990).

Many lines of research suggest that few unassembled nuclear-encoded proteins are allowed to accumulate in chloroplasts. For instance, when the chloroplast-directed synthesis of the large subunit of ribulose bisphosphate carboxylase/oxygenase is inhibited, newly imported small subunits which are not assembled into the enzyme complex have a half-life of <8 min in the stroma (Schmidt and Mishkind, 1983). Similarly, when the copper insertion reaction of apoplastocyanin is inhibited in *Chlamydomonas reinhardtii* by growing the cells in copper-deficient media, the apoprotein is still targeted correctly, but is rapidly degraded in the thylakoid lumen (Merchant and Bogorad, 1986; but see Bohner et al., 1981). Other polypeptides appear to be rapidly turned over when not stabilized by binding to chlorophyll *a* (Mullet et al., 1990; Bennett, 1981), or by assembly into the PS II reaction center (Rochaix and Erickson, 1988; Nilsson et al., 1990). This recurrent theme, rapid degradation of unassembled nuclear-encoded subunits and apoproteins, suggests that the turnover of excess subunits helps to ensure the correct stoichiometric balance between polypeptides in a protein complex (Schmidt and Mishkind, 1983; Biekmann and Feierabend, 1985; Luzikov, 1986).

In contrast, the polypeptides of the OEC do not appear to

1. *Abbreviations used in this paper*: OEC, oxygen-evolving complex; OE33, OE23, and OE17, the 33-, 23-, and 17-kD subunits of the OEC, respectively; PS II, photosystem II; PC and prPC, mature and precursor forms of plastocyanin, respectively.

follow this general rule, as many mutations affecting their assembly with the PS II reaction center allow the unassembled OEC subunits to accumulate (reviewed in Rochaix and Erickson, 1988; see also, Mayfield et al., 1989; de Vitry et al., 1989; Nilsson et al., 1990). The unassembled polypeptides are apparently also stable in hydroxylamine-treated leaf segments, in wheat leaves grown in intermittent light, and in etiolated barley (Becker et al., 1985; Callahan and Chenaie, 1985; Ono et al., 1986; Ryrie et al., 1984). While these results suggest that the unassembled OEC polypeptides can be relatively stable under certain nonphysiological conditions, their accumulation in normal physiologically active chloroplasts has not been addressed before.

Our laboratory is interested in the import, targeting, and assembly in isolated intact chloroplasts of the extrinsic subunits of the OEC using in vitro synthesized polypeptide. Unexpectedly, sub-fractionation of pea chloroplasts after import reactions consistently revealed that a large fraction of the newly imported (radiolabeled) OEC polypeptides were not associated with the thylakoid membranes, but rather remained in a soluble lumen fraction (data not shown). This raised the question of whether OEC subunits were dissociated from the complex by the fractionation conditions used, or whether they had not assembled. To address this question, we determined the lowest concentrations of Triton X-100 required to gently rupture the pea thylakoids and release the lumen contents while retaining the OEC entirely intact. The results presented here show for the first time that a significant portion of the OEC polypeptides present in normal chloroplasts that have not undergone any inhibitory treatments are free in the lumen and may be released by as little as 0.04% Triton X-100. Examination of the rates of oxygen evolution from the resulting membrane fragments demonstrated that these detergent concentrations did not cause the dissociation of the extrinsic subunits from active OECs. Similar, though smaller, pools of unassembled subunits were detected in thylakoids derived from spinach chloroplasts. Our data indicate that, unlike the other polypeptides described above, newly targeted, unassembled subunits of the OEC are not rapidly degraded inside the chloroplasts. These findings extend to fully greened, physiologically healthy chloroplasts the notion derived from studies with nonphysiological samples (Becker et al., 1985; Callahan and Cheniae, 1985; Ono et al., 1986) that pools of unassembled subunits may function in the biogenesis and/or homeostasis of the OEC.

Materials and Methods

Chloroplast, Thylakoid, and PS II Particle Isolation

Peas (*Pisum sativum* cv Progress #9) were grown in moist vermiculite in a greenhouse or in the laboratory for 10–16 d; spinach (*Spinacia oleracea*) was purchased from a local market. Intact chloroplasts and thylakoids were isolated essentially as described by Cline et al. (1985) and by Theg et al. (1986), respectively. Photosystem II particles were isolated from purified thylakoids by Triton X-100 extraction as described by Berthold et al. (1981), except that the extraction buffer contained 400 mM sucrose, 50 mM Na-Mes at pH 6.5, 10 mM NaCl, and 5 mM $MgCl_2$ (Dennenberg et al., 1986).

Synthesis and Import of prPC

Transcriptions from the linearized plasmid containing a cDNA clone for the precursor of plastocyanin (prPC) were performed with SP6 RNA polymerase; translation of the resulting mRNA was carried out in the presence of [^{3}H]leucine (NEN) in a wheat germ lysate (Theg et al., 1989). Import reactions were performed in 60 μl by incubating radiolabeled prPC (10^6 DPM) with freshly isolated chloroplasts (20 μg chlorophyll) in import buffer (50 mM K-Tricine pH 8.0, 0.33 M sorbitol) supplemented with 3 mM ATP and 5 mM $MgCl_2$ for 20 min, after which the intact chloroplasts were repurified through 40% Percoll (Cline et al., 1985). The repurified intact chloroplasts were then analyzed without further treatment, or subjected to fractionation as described below.

Triton X-100 Treatment of Thylakoids

Intact chloroplasts were washed once in import buffer to remove traces of Percoll, and then osmotically lysed in 10 mM Na-Hepes pH 6.5, 5 mM $MgCl_2$ on ice for 3 min. Thylakoids were collected by pelleting at 4,000 *g* for 5 min, and the supernatant (stromal fraction) was saved for analysis. The thylakoids were resuspended in a small volume of thylakoid buffer (400 mM sucrose, 50 mM Na-Mes at pH 6.5, 10 mM NaCl, and 5 mM $MgCl_2$), then gently combined with an equal volume of thylakoid buffer containing twice the indicated percent (wt/vol) of Triton X-100. After 3 min on ice, the samples were centrifuged at 100,000 *g* for 8 min in a TL-100.3 rotor to separate thylakoids from the supernatant (lumen fraction). Fractions of samples were subjected to SDS-PAGE and prepared for fluorography or Western blotting, or were analyzed for oxygen evolution and chlorophyll content.

Isolation of OEC Polypeptides and Production of Antibodies

Extrinsic OEC polypeptides were extracted from PS II particles by alkaline

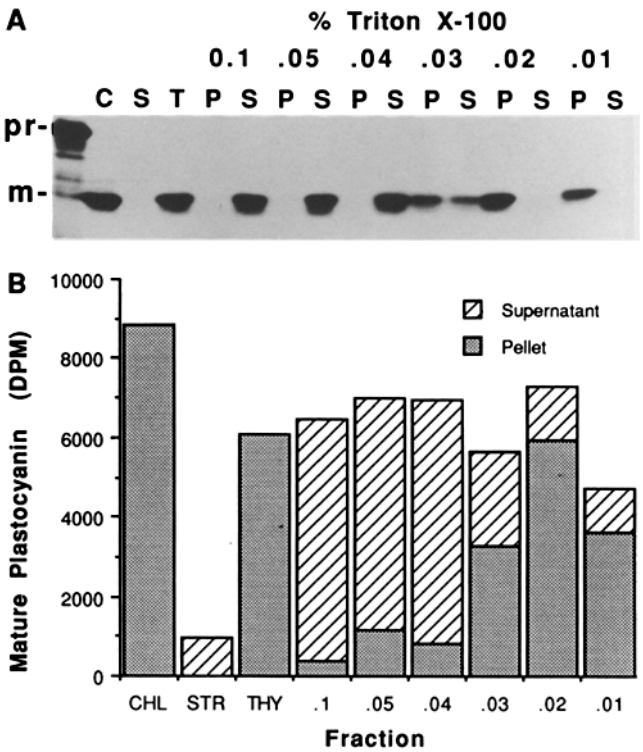

Figure 1. Release of newly imported PC from the lumen of pea thylakoids by Triton X-100 treatment. After import of prPC, chloroplasts (*C*) were lysed and the stroma (*S*) and thylakoid (*T*) fractions recovered. The thylakoids were then resuspended and mixed with an equal volume of buffer containing twice the indicated amount (% wt/vol) of Triton X-100. The samples were fractionated by centrifugation at 100,000 *g* for 8 min, and the supernatant (*S*) and pellet (*P*) fractions were analyzed by SDS-PAGE and prepared for fluorography. (*A*) fluorograph; *pr*− and *m*− mark the positions of the precursor and mature forms of PC, respectively. (*B*) Quantitation of the fluorograph.

Tris washing (Yamamoto et al., 1971). The Tris-released polypeptides were precipitated with an excess of 1.5 M perchloric acid, separated by SDS-PAGE on a preparative 15% gel, and visualized by staining with Coomassie blue R-250. The protein-containing bands were excised and stored at −80°C until used. Gel slices were prepared for immunization by crushing the gel in an equal volume of TBS (Harlow and Lane, 1988), followed by homogenization with another equal volume of a 1:1 mixture of complete and incomplete Freunds's adjuvants (first injection), or an equal volume of incomplete Freunds's adjuvant (subsequent injections). Chickens were immunized twice (OE33) or three times (OE23 and OE17) by multiple intramuscular injections at 1 wk intervals. IgG was prepared from egg yolk as described (Polson, 1980).

Oxygen Evolution Measurements

The rates of oxygen evolution from samples containing 50 μg chlorophyll were measured with a Clark-type oxygen electrode in a solution containing 200 mM sucrose, 25 mM Na-Mes pH 6.5, 5 mM NaCl, 2.5 mM $MgCl_2$, 2 μM nigericin, and 200 μM 2,6-dichloro-*p*-benzoquinone/1 mM $K_3Fe(CN)_6$ as an electron acceptor pair. Salt-washed PS II particles used in Fig. 4 were prepared by incubating samples in extraction buffer (400 mM Sucrose, 50 mM Na-Mes pH 6.5, 5 mM $MgCl_2$) containing 2 M NaCl (salt washed) or 15 mM NaCl (control). After 30 min on ice the PS II particles were pelleted again and resuspended in extraction buffer containing 15 mM NaCl. Preincubation with hydroquinone was performed in 3 mM hydroquinone in the dark on ice for 30 min.

Miscellaneous

SDS-PAGE in either 12.5 or 15% gels was performed as described (Laemmli, 1970). Proteins were visualized by fluorography (Cline et al., 1985) or by Western blotting (Towbin et al., 1979) and probed with alkaline phosphatase–conjugated goat anti-rabbit or goat anti-chicken antibodies in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Blake et al., 1984). Fluorographs were quantitated by scintillation counting of excised gel slices (Olsen et al., 1989), and quantitation of Western blots was performed with a Bio Image analyzer using Visage 4.5 software (BioImage Products, Ann Arbor, MI). All reagents and enzymes were purchased from commercial vendors. The plasmid containing prPC was a gift from Drs. J. C. Gray and D. I. Last (University of Cambridge), and the antibody raised to the maize OE17 was a gift from Dr. R. T. Sayre (Ohio State University).

Results

Gentle Disruption of the Thylakoid Membrane Causes the Release of OEC Subunits from the Lumen

Our laboratory is studying the pathway of assembly of the OEC from newly imported nuclear-encoded subunits. Our initial experiments in which we fractionated the chloroplasts and thylakoids after an import reaction, revealed that a large portion of the newly imported OEC subunits remained unassembled in the thylakoid lumen (data not shown). To explain these results we postulated that the 1% Triton X-100 used to fractionate the thylakoids had caused the dissociation of some OECs, even though this detergent is routinely used at a higher concentration to prepare oxygen-evolving PS II particles (Berthold et al., 1981). This postulate led us to determine the lowest concentration of Triton X-100 required to release the contents of the thylakoid lumen into the medium.

Fig. 1 shows a fluorograph from an experiment in which we monitored the location of newly imported radiolabeled PC after fractionation of the thylakoids with different concentrations of Triton X-100. PC was chosen as a control because it is not generally associated with the thylakoid membrane, but rather remains free in the lumen (Haehnel et al., 1989). After incubation of thylakoids with Triton X-100 concentrations higher than 0.03%, we found that PC did not pellet with the membranes, indicating that it had been released from the lumen by the detergent. PC pelleted with thylakoids treated with detergent concentrations <0.02%, which is approximately the critical micelle concentration under our conditions (Helenius and Simmons, 1975). At 0.03% Triton X-100, PC was split between the pellet and supernatant, indicating that this concentration of detergent was not enough to completely open the thylakoid vesicles. These results indicate that 0.04% is the lowest concentration of Triton X-100 that will cause the complete release of the contents of the thylakoid lumen to the medium. None of the detergent concentrations used in this experiment solubilized significant amounts of chlorophyll from the thylakoid membranes (data not shown).

The low levels of Triton X-100 required to free PC from the lumen also caused the release of a fraction of the OEC polypeptides from the thylakoids. Fig. 2 shows a Western blot detecting soluble and membrane-bound OEC polypeptides after Triton X-100 treatment of thylakoids. A discrete fraction of the total complement of OEC polypeptides followed PC during fractionation of the thylakoids with detergent; OEC subunits were detected in the soluble fraction after treatment with concentrations of detergent between 1.0 and 0.03%, and remained with the membranes at detergent concentrations of 0.02% and below. Quantitation of the amount of OEC polypeptides in the soluble and membrane bound fractions showed some variability from one experiment to the next, but revealed that between 10 and 20% of OE33, 40 and 60% of OE23, and 15 and 50% of OE17 were released from the lumen by treatment with 0.04% Triton X-100. We would emphasize again that the intact chloroplasts used in these experiments had never undergone any inhibitory treatments that might lead to the release of the OEC subunits from the membranes.

Low Concentrations of Triton X-100 Do Not Solubilize the OEC

Two possibilities existed to explain the appearance of OEC subunits in the lumen fraction of thylakoids treated with low concentrations of Triton X-100. The first is that these detergent concentrations, even though >100-fold lower than normally used to prepare oxygen-evolving PS II particles, were still sufficient to dissociate some of the functional OECs from the thylakoid membrane. Alternatively, the soluble OEC polypeptides might have already been present in the lumen in unassembled pools of subunits at the time the chloroplasts were isolated from the leaves.

We tested this first possibility by examining the ability of detergent-treated thylakoids to evolve oxygen. We reasoned that if Triton X-100 treatment of the thylakoids did dissociate active OECs, the oxygen evolution rates should be adversely effected, and the extent of this effect should correlate roughly with the amounts of subunits released. In the experiment shown in Fig. 3 we measured the rate of oxygen evolution from control and detergent-treated samples. The samples were derived from an experiment similar to that shown in Fig. 2 in which ~40% of OE23 was released by incubation with 0.04% Triton X-100. As can be seen in the figure, treatment of thylakoids with up to 0.1% Triton X-100 did not result in a significant decrease in the ability of the samples to evolve oxygen; certainly nothing approaching a 40% decline in activity was observed.

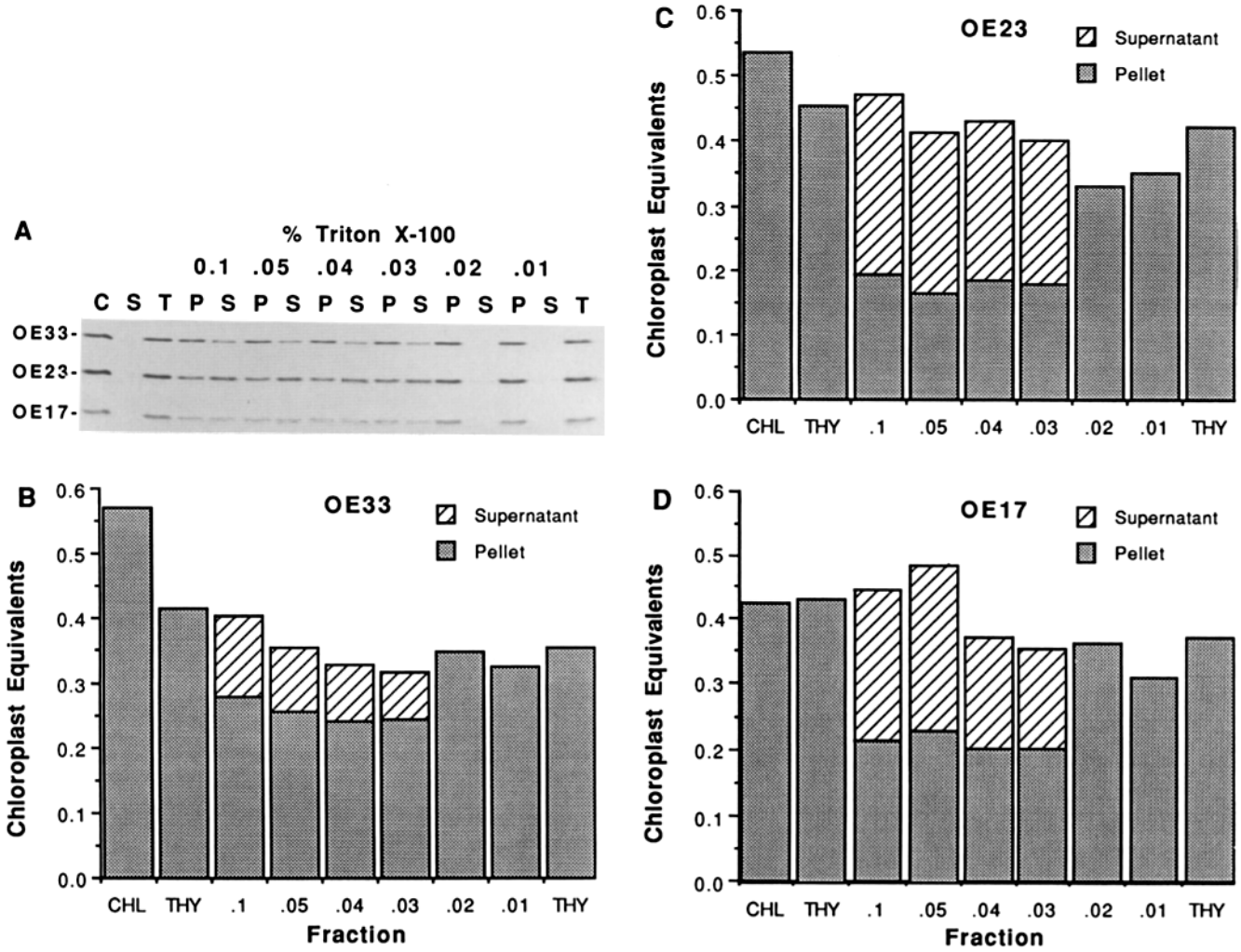

Figure 2. Release of OEC polypeptides from the lumen of pea thylakoids by Triton X-100 treatment. Proteins in the chloroplast (*C*), stroma (*S*), thylakoid (*T*), pellet (*P*), and supernatant (*S*) fractions derived as in Fig. 1 were fractionated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was reacted with a combination of anti-OE33, anti-OE23, and anti-OE17 followed by alkaline phosphatase-conjugated secondary antibody. Dilutions of purified intact pea chloroplasts were used as standards for the quantitation of the Western blot; quantified bands were within the linear scale of the standards. The quantified results are expressed in units of chloroplast equivalents, defined for each individual protein as the amount of that protein present in a preparation of intact chloroplasts containing 1 μg of chlorophyll. (*A*) Western blot; (*B*) quantitation of OE33; (*C*) quantitation of OE23; (*D*) quantitation of OE17.

It has long been recognized that PS II particles that have been depleted of OE23 and OE17 by washing with 2 M NaCl are still capable of evolving oxygen provided that high concentrations of Ca^{2+} and Cl^- are present in the assay medium (Ghanotakis et al., 1984*a*). Similarly, NaCl-washed PS II particles are susceptible to inhibition by bulky reductants such as hydroquinone, whereas the control samples are not (Ghanotakis et al., 1984*b*). Thus, both an unusual requirement for Ca^{2+} and Cl^- for oxygen evolution and a sensitivity of the reaction to hydroquinone are diagnostic of OECs from which some of the extrinsic subunits have been dissociated. We used these tests to further confirm that the detergent treatment applied in our experiments did not affect the integrity of the OEC.

Fig. 4 *A* shows the expected stimulation of oxygen evolution from NaCl-washed PS II particles by $CaCl_2$. This figure also documents the expected inhibition of oxygen evolution from the same samples by pretreatment with hydroquinone. Fig. 4 *B* demonstrates that oxygen evolution from thylakoids treated with low concentrations of Triton X-100 were neither stimulated by $CaCl_2$ nor inhibited by hydroquinone. These results support the conclusions already reached from Fig. 3; the OEC polypeptides released by low Triton X-100 concentrations were not dissociated from the active complex by the detergent, but rather were present from the start of our experiments in pools of unassembled subunits in the thylakoid lumen.

Pools of Unassembled OEC Subunits Are Also Present in the Lumen of Thylakoids Derived from Spinach Chloroplasts

The experiments reported above were performed with pea chloroplasts. In fact, oxygen-evolving PS II particles derived

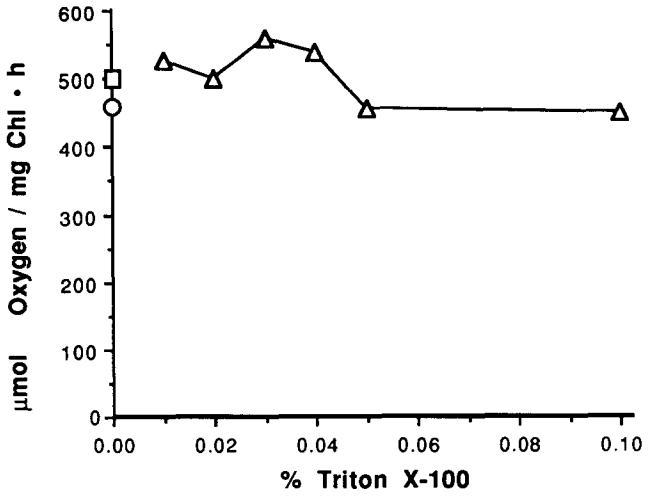

Figure 3. Rates of oxygen evolution from thylakoid and pellet fractions of detergent-treated pea thylakoids. (○) Intact chloroplasts before envelope lysis; (□) thylakoids before detergent treatment; (△) pellet fraction of thylakoids treated with Triton X-100. Samples were derived from the experiment shown in Fig. 1.

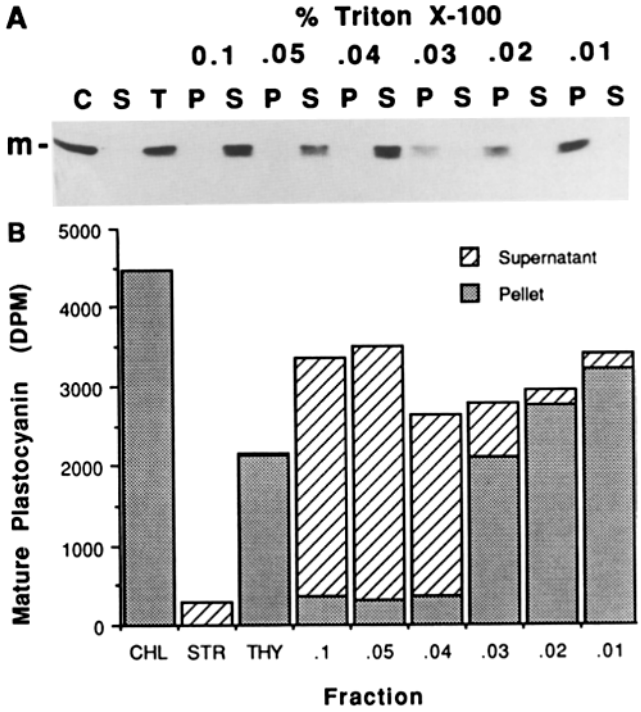

Figure 5. Release of newly imported PC from the lumen of spinach thylakoids by Trion X-100 treatment. The experiment was performed as described for peas in Figure 1. (*A*) Fluorograph; m^- marks the position of mature PC. (*B*) Quantitation.

from spinach chloroplasts are somewhat better characterized than those from peas. Since the pools of OEC subunits identified by us have not been reported in spinach thylakoids, we questioned whether they might only be present in pea chloroplasts. This is not the case, however, since we detected similar, though smaller, pools of unassembled subunits in spinach thylakoids. Thylakoids from both species required ~0.04% Triton X-100 to release newly imported PC from the lumen (compare Figs. 1 and 5). Fig. 6 shows that, like pea thylakoids, the lumen of spinach thylakoids contained a significant fraction of the total OEC subunits in an unassembled pool (8, 30, and 30% for OE33, OE23, and OE17, respectively). Measurements of oxygen evolution from detergent-treated spinach thylakoids (analogous to Fig. 3) revealed that the pools were not due to dissociation of the OEC by Triton X-100 (data not shown). Thus, the presence of pools of unassembled OEC polypeptides in the lumen of thylakoids before chloroplast isolation appears to be a general phenomenon, found in both spinach and peas. This suggests that the pools may play an important role in OEC biogenesis or homeostasis in green plants.

Discussion

The synthesis, targeting, maturation, and assembly of multimeric protein complexes in organelles is a complex process, and the biogenesis of the OEC is no exception. The OEC subunits are encoded in the nucleus and translated in the cytosol (Ghanotakis and Yocum, 1990). From the cytosol they are bound by and then translocated into chloroplasts,

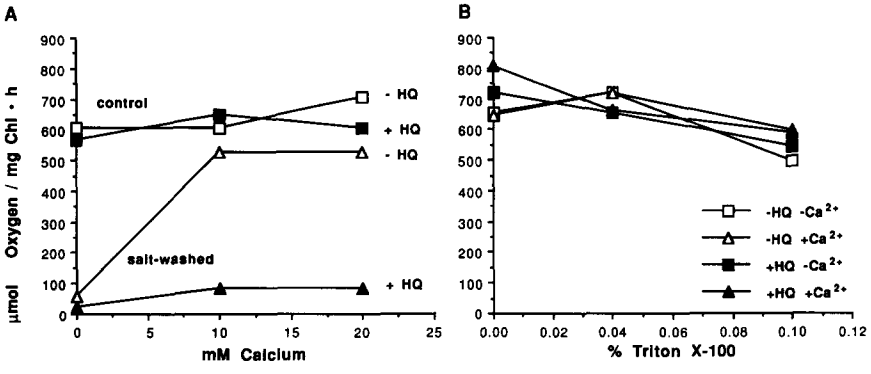

Figure 4. Effects of hydroquinone pretreatment and Ca^{2+} on oxygen evolution rates from salt-washed PS II particles (*A*), and on Triton X-100 treated thylakoids (*B*). (*A*) PS II particles were washed with 2 M NaCl to remove OE23 and OE17. Where indicated, portions of the control and salt-washed particles were preincubated with hydroquinone as described in Materials and Methods. (*Squares and triangles*) Control and salt-washed PS II particles, respectively. (*Open symbols*) No hydroquinone pretreatment; closed symbols, samples preincubated with hydroquinone. (*B*) Thylakoids were incubated with Triton X-100 as in the experiment described in Fig. 1. Where indicated, pellet fractions were preincubated with hydroquinone. (*Squares*) No Ca^{2+}; (*triangles*) + 10 mM Ca^{2+}. (*Open symbols*) No hydroquinone pretreatment; (*closed symbols*) samples preincubated with hydroquinone.

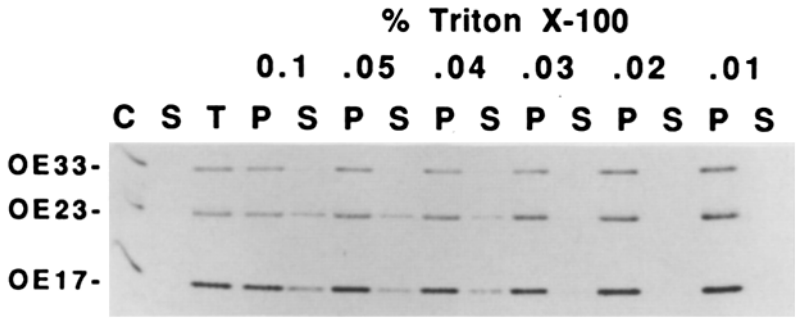

Figure 6. Release of OEC polypeptides from the lumen of spinach thylakoids by Triton X-100 treatment. Proteins from total chloroplast (*C*), total thylakoid (*T*), stroma (*S*), and in the pellet (*P*) and supernatant (*S*) fractions from detergent-treated spinach thylakoids from the experiment described in Fig. 5 were fractionated and probed as in Fig. 2, except that the spinach OE17 was identified by an antibody raised against maize OE17 (anti-pea OE17 did not cross-react with the spinach OE17). Quantitation of the blot (as described in Fig. 2, but using purified spinach chloroplasts as standards) revealed that 8% of OE33, 30% of OE23, and 30% of OE17 was free in the thylakoid lumen.

presumably first into the stroma (James et al., 1989). Both the binding and translocation steps require ATP (Olsen et al., 1989; Theg et al., 1989). The subunits are thought to undergo proteolytic processing in the stroma, resulting in intermediate-sized proteins that are the substrates for subsequent translocation across the thylakoid membrane. Finally, the polypeptides are processed in the thylakoid lumen to their mature sizes, and then assembled into the membrane-associated OEC. The roles, if any, of cytoplasmic or stromal factors, or of thylakoid membrane heterogeneity, in the different translocation and assembly steps remain to be elucidated, but may be inferred from studies of other plastid proteins (Pain et al., 1988; Cline et al., 1989; Yalovsky et al., 1990).

In this communication we have addressed one aspect of the assembly of the OEC, namely the functional sites from which subunits for new assembly are likely to be chosen. Experiments with a number of other nuclear-encoded chloroplast proteins have suggested that, as a general rule, they are either assembled immediately upon import from the cytoplasm, or they are rapidly degraded. This is shown schematically in Fig. 7 *A*. 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, CP43 and subunits of the chloroplast coupling factor (Schmidt and Mishkind, 1983; Bennett, 1981; Mullet et al., 1990; Biekmann and Feierabend, 1985). It has been suggested that this mechanism may help to ensure the proper balance between subunits derived from the different genomes (Schmidt and Mishkind, 1983; Biekmann and Feierabend, 1985; Luzikov, 1986). Our experiments indicate that the OEC subunits apparently do not conform to this general rule. Instead of being rapidly degraded, the newly imported (and presumably in vivo, newly synthesized) unassembled polypeptides appear to enter a pool containing a considerable fraction of the total chloroplast complement of OEC subunits. From here, they are presumably chosen at random for synthesis of new complexes. This pathway is depicted schematically in Fig. 7 *B*.

Although our experiments provide the first direct evidence for the presence of pools of unassembled OEC subunits in physiologically active chloroplasts, this notion can be in-

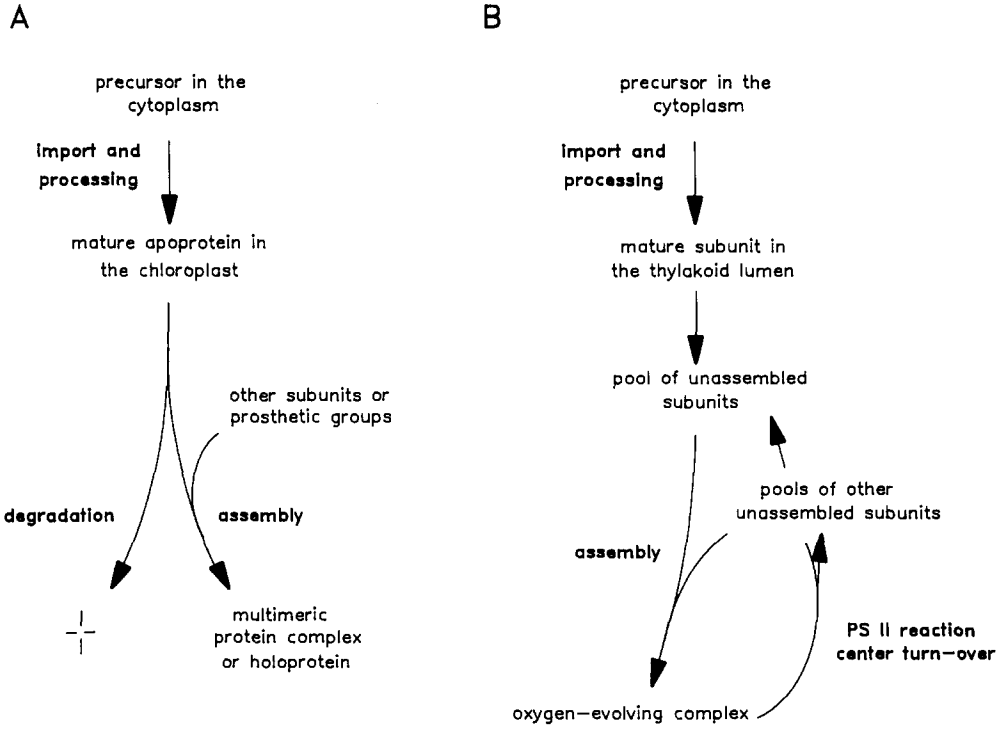

Figure 7. Pathways for the assembly of chloroplast protein complexes. (*A*) Assembly pathway apparently used by most plastid protein complexes and apoproteins. (*B*) Proposed pathway for the assembly of the OEC.

ferred from earlier published work on mutant, inhibited or greening plastids. A number of mutants with defects in the PS II reaction center core have been described. These mutants, which do not assemble the core complex, are often found to contain relatively normal amounts of OEC subunits, even though they are unable to assemble them with PS II (reviewed in Rochaix and Erickson, 1988; see also, Mayfield et al., 1989; de Vitry et al., 1989; Nilsson et al., 1990). Similarly, etiolated plastids in barley which do not contain a functional PS II core accumulate normal levels of OE33 prior to greening (Ryrie et al., 1984). In addition, when the OEC is dissociated in intact thylakoids by a number of treatments, the OEC subunits do not appear to be rapidly degraded, and in fact may be used in subsequent reconstitution steps (Becker et al., 1985; Callahan and Chenaie, 1985). These experiments performed with inhibited or damaged chloroplasts, while not addressing the existence of pools of unassembled OEC subunits directly, suggest at least that the unassembled polypeptides are not subject to rapid proteolysis.

The discussion above points out that unassembled subunits of chloroplast protein complexes are not usually allowed to accumulate. This apparently is also true for other protein complexes throughout the cell. Luzikov (1986) has presented a hypothesis that attempts to provide a link between the fate of unassembled subunits and protein complex assembly. He proposes that proteins destined for assembly into multimeric complexes are "hyperexpressed", and that selective degradation of those subunits which are not correctly targeted or assembled results in a high proportion of properly assembled complexes, each with the correct subunit stoichiometry. This model was based upon consideration of the assembly of numerous protein complexes in different locations within the cell (see also the discussion by Hare, 1990). Nevertheless, some exceptions to this general trend have been reported; a few subunits of the *Neurospora crassa* mitochondrial NADH dehydrogenase (Videira and Werner, 1989) and cytochrome oxidase (Schwab et al., 1972) appear to accumulate up to a level 25% higher than that expected by their assembled stoichiometries. The data from other laboratories (Rochaix and Erickson, 1988; see also, Mayfield et al., 1989; de Vitry et al., 1989; Nilsson et al., 1990) as well as the results we report here place the OEC in this list of exceptional protein complexes whose unassembled subunits are not rapidly degraded.

From this point of view, the question arises as to why such an unusual assembly pathway might be used. The most obvious answer lies in the rapid turnover of D1, a protein of the PS II reaction center core. This protein is subjected to continuous swift light-dependent degradation, which presumably results in destabilization of the entire core complex (Ohad et al., 1985; Virgin et al., 1990; reviewed in Mattoo et al., 1989). Recent experiments by Andersson and his colleagues (Hundal et al., 1990) have demonstrated that the OEC polypeptides dissociate from the PS II core as a result of light-induced D1 turnover. These and other authors (Becker et al., 1985; Callahan and Cheniae, 1985) suggested that the dissociated subunits can be reused in the assembly of new OECs upon regeneration of PS II. However, a number of lines of research indicate that the pools of OEC subunits described in this work are not derived solely from proteins released during inhibition of PS II. First, the chloroplasts we observed to contain the subunit pools were not subjected to any inhibitory treatments. On the contrary, they were kept on ice in the dark until use as a means to maximally preserve their intrinsic activities. Second, radiolabeled OEC subunits imported in vitro into isolated chloroplasts were found uncomplexed in the thylakoid lumen (data not shown). Finally, the relative amounts of the subunits observed in the soluble pools did not reflect the 1:1:1 stoichiometry of the OEC (see Figs. 2 and 5), as would have been expected if they were derived from functional complexes. These observations lend support to the model, depicted in the scheme of Fig. 7 *B*, that OEC subunits enter the soluble pools in the lumen by two routes; after their release from active complexes during inhibition, and by the import of newly synthesized polypeptides. The presence of readily available OEC polypeptides in the thylakoid lumen would presumably be advantageous during the (re)synthesis of PS II reaction centers since photoinactivation of the centers occurs rapidly when the OEC has been disabled (Yamashita and Butler, 1969; Cheniae and Martin, 1971; Callahan and Chenaie, 1985; Theg et al., 1986; Callahan et al., 1986; Jegerschoeld et al., 1990).

The results presented here suggest that the assembly of newly synthesized and imported OEC polypeptides into active enzyme complexes is likely to be influenced by several factors. Among these is the rate of exchange, if any, between the assembled and unassembled subunits, the rate of turnover of the subunits in the pools, and the rate of turnover and resynthesis of PS II core proteins. We are currently assessing the stability of the pools and trying to determine what factors affect pool utilization. The ability to follow the assembly of radiolabeled precursors into active oxygen-evolving complexes in organello should yield new insights into the processes that guide the assembly of the OEC.

We wish to thank Dr. Kirk Klasing for providing the chickens and facilities used for the preparation of the anti-OEC antibodies, and Drs. C. F. Yocum and N. R. Bowlby for valuable discussions during the initial stages of these experiments.

This work was supported by National Science Foundation grant DCB 88-17373 to S. M. Theg.

Received for publication 11 April 1991 and in revised form 25 June 1991.

References

Babcock, G. T. 1987. The photosynthetic oxygen-evolving process. *In* Photosynthesis. J. Amez, editor. Elsevier, Amsterdam. 125–158.

Becker, D. W., F. E. Callahan, and G. M. Chenaie. 1985. Photoactivation of NH_2OH-treated leaves: reassembly of released extrinsic PS II polypeptides and religation of the polynuclear Mn catalyst of water oxidation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 192:209–214.

Bennett, J. 1981. Biosynthesis of the light-harvesting chlorophyll a/b protein: polypeptide turnover in darkness. *Eur. J. Biochem.* 118:61–70.

Berthold, D. A., G. T. Babcock, and C. F. Yocum. 1981. A highly resolved oxygen-evolving photosystem II preparation from spinach thylakoid membranes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 134:231–234.

Biekmann, S., and J. Feierabend. 1985. Synthesis and degradation of unassembled polypeptides of the coupling factor of photophosphorylation CF1 in 70S ribosome-deficient rye leaves. *Eur. J. Biochem.* 152:529–535.

Blake, M. S., K. H. Johnson, G. J. Russel-Jones, and E. C. Gotschlich. 1984. A rapid sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* 136:175–179.

Bohner, H., H. Boehme, and P. Boeger. 1981. Biosynthesis of plastocyanin: identification of precursors. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 131:186–388.

Burnap, R. L., and L. A. Sherman. 1991. Deletion mutagenesis in *Synechocystis* sp. PCC6803 indicates that the Mn-stabilizing protein of photosystem II is not essential for O_2 evolution. *Biochemistry.* 30:440–446.

Callahan, F. E., and G. M. Chenaie. 1985. Studies on the photoactivation of the water oxidizing enzyme. I. Processes limiting photoactivation in hydrox-

ylamine-extracted leaf segments. *Plant Physiol.* 79:777–786.
Callahan, F. E., D. W. Becker, and G. M. Chenaie. 1986. Studies on the photoactivation of the water oxidizing enzyme. II. Characterization of weak light photoinhibition and its light induced recovery. *Plant Physiol.* 82:261–269.
Cheniae, G. M., and I. F. Martin. 1971. Effects of hydroxylamine on photosystem II. I. Factors affecting the decay of O_2 evolution. *Plant Physiol.* 47:568–575.
Cline, K., M. Werner-Washburn, T. H. Lubben, and K. Keegstra. 1985. Precursors to two nuclear encoded chloroplast proteins bind to the outer envelope membrane before being imported into chloroplasts. *J. Biol. Chem.* 260:3691–3696.
Cline, K., D. R. Fulsom, and P. V. Viitanen. 1989. An imported thylakoid protein accumulates in the stroma when insertion into thylakoid membranes is inhibited. *J. Biol. Chem.* 264:14225–14232.
Dennenberg, R. J., P. A. Jursinic, and S. A. McCarthy. 1986. Intactness of the oxygen-evolving system in thylakoids and photosystem II particles. *Biochim. Biophys. Acta.* 852:222–233.
de Vitry, C., J. Olive, D. Drapire, M. Recouvreur, and F.-A. Wollman. 1989. Posttranslational events leading to the assembly of photosystem II protein complex: a study using photosynthesis mutants from *Chlamydomonas reinhardtii*. *J. Cell Biol.* 109:991–1006.
Ghanotakis, D. F., G. T. Babcock, and C. F. Yocum. 1984*a*. Calcium reconstitutes high levels of oxygen evolution in polypeptide-depleted photosystem II preparations. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 167:127–130.
Ghanotakis, D. F., J. N. Topper, and C. F. Yocum. 1984*b*. Structural organization of the oxidizing side of photosystem II: exogenous reductants reduce and destroy the Mn-complex in photosystem II membranes depleted of the 17 and 23 kDa polypeptides. *Biochim. Biophys. Acta.* 767:524–531.
Ghanotakis, D. F., and C. F. Yocum. 1990. Photosystem II and the oxygen-evolving complex. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:255–276.
Haehnel, W., R. Ratajczak, and H. Robenek. 1989. Lateral distribution and diffusion of plastocyanin in chloroplast thylakoids. *J. Cell Biol.* 108:1397–1405.
Hare, J. F. 1990. Mechanisms of membrane protein turnover. *Biochim. Biophys. Acta.* 1031:71–90.
Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 726 pp.
Helenius, A., and K. Simons. 1975. Solubilization of membranes by detergents. *Biochim. Biophys. Acta.* 415:29–79.
Hundal, T., I. Virgin, S. Styring, and B. Andersson. 1990. Changes in the organization of photosystem II following light-induced D1 protein degradation. *Biochim. Biophys. Acta.* 1017:235–241.
James, H. E., D. Bartling, J. E. Musgrove, P. M. Kirwin, and C. Robinson. 1989. Transport of proteins into chloroplasts: import and maturation of precursors to the 33-, 23-, and 16-kDa proteins of the photosynthetic oxygen-evolving complex. *J. Biol. Chem.* 264:19573–19576.
Jegerschoeld, C., I. Virgin, and S. Styring. 1990. Light dependent degradation of the D1 protein in photosystem II is accelerated after inhibition of the water splitting reaction. *Biochemistry.* 29:6179–6186.
Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
Luzikov, V. N. 1986. Proteolytic control over topogenesis of membrane proteins. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 200:259–264.
Mattoo, A. K., J. B. Marder, and M. Edelman. 1989. Dynamics of the photosystem II reaction center. *Cell.* 56:241–246.
Mayfield, S. P., M. Schirmer-Rahire, F. Gerhard, H. Zuber, and J. D. Rochaix. 1989. Analysis of the genes of the OEE1 and OEE3 proteins of the photosystem II complex from *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* 12:683–693.
Merchant, S., and L. Bogorad. 1986. Rapid degradation of apoplastocyanin in Cu(II) deficient cells of *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 261: 15850–15853.
Miyao, M., and N. Murata. 1989. The mode of binding of three extrinsic proteins of 33 kDa, 23 kDa, and 18 kDa in the photosystem II complex of spinach. *Biochim. Biophys. Acta.* 977:315–321.
Mullet, J. E., P. G. Klein, and R. R. Klein. 1990. Chlorophyll regulates accumulation of the plastid encoded chlorophyll apoproteins CP43 and D1 by increasing apoprotein stability. *Proc. Natl. Acad. Sci. USA.* 87:4038–4042.
Nilsson, F., B. Andersson, and C. Jansson. 1990. Photosystem II characteristics of a constructed *Synechocystis* 6803 mutant lacking synthesis of the D1 polypeptide. *Plant Mol. Biol.* 14:1051–1054.
Ohad, I., D. J. Kyle, and J. H. Hirschberg. 1985. Light-dependent degradation of the QB-protein in isolated pea thylakoids. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1655–1659.
Olsen, L. J., S. M. Theg, B. R. Selman, and K. Keegstra. 1989. ATP is required for the binding of precursor proteins to chloroplasts. *J. Biol. Chem.* 246:6724–6729.
Ono, T.-A., H. Kaijikawa, and Y. Inoue. 1986. Changes in protein composition and Mn abundance in Photosystem II particles on photoactivation of the latent O_2-evolving system in flash-grown wheat leaves. *Plant Physiol.* 80:85–90.
Pain, D., Y. S. Kanwar, and G. Blobel. 1988. Identification of a receptor for protein import into chloroplasts and its localization to envelope contact sites. *Nature (Lond.).* 331:232–237.
Polson, A., M. B. von Wechmar, and G. Fazakerley. 1980. Antibodies to proteins from yolk of immunized hens. *Immunol. Commun.* 9:495–514.
Rochaix, J. D., and J. Erickson. 1988. Function and assembly of photosystem II: genetic and molecular analysis. *Trends Biochem. Sci.* 13:56–59.
Ryrie, I. J., S. Young, and B. Andersson. 1984. Development of the 33-, 23-, and 16-kDa polypeptides of the photosynthetic oxygen-evolving system during greening. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 177:269–273.
Schmidt, G. W., and M. L. Mishkind. 1983. Rapid degradation of unassembled ribulose 1,5-bisphosphate carboxylase small subunits in chloroplasts. *Proc. Natl. Acad. Sci. USA.* 80:2632–2636.
Schwab, A. J., W. Sebald, and H. Weiss. 1972. Different pool sizes of the precursor polypeptides of cytochrome oxidase from *Neurospora crassa*. *Eur. J. Biochem.* 30:511–516.
Theg, S. M., L. J. Filar, and R. A. Dilley. 1986. Photoinactivation of chloroplasts already inhibited on the oxidizing side of photosystem II. *Biochim. Biophys. Acta.* 849:104–111.
Theg, S. M., C. Bauerle, L. J. Olsen, B. R. Selman, and K. Keegstra. 1989. Internal ATP is the only energy requirement for the translocation of precursor proteins across chloroplast membranes. *J. Biol. Chem.* 264:6730–6736.
Towbin, H., T. Stachelin, and G. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
Videira, A., and S. Werner. 1989. Assembly kinetics and identification of precursor proteins of complex I from *Neurospora crassa*. *Eur. J. Biochem.* 181:493–502.
Virgin, I., D. F. Ghanotakis, and B. Andersson. 1990. Light-induced D1-protein degradation in isolated photosystem II core complexes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 269:45–48.
Yalovsky, S., G. Schuster, and R. Nechustai. 1990. The apoprotein precursor of the major light harvesting complex of photosystem II (LHC-IIB) is inserted primarily into stroma lamellae and subsequently migrates into the grana. *Plant Mol. Biol.* 14:753–764.
Yamamoto, Y., M. Doy, N. Tamura, and M. Nishimura. 1971. Release of polypeptides from highly active O_2 evolving photosystem II preparations by tris treatment. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 133:265–268.
Yamashita, T., and W. L. Butler. 1969. Photooxidation by photosystem II of Tris-washed chloroplasts. *Plant Physiol.* 44:1342–1346.