

# Evidence for Two-step Processing of Nuclear-encoded Chloroplast Proteins during Membrane Assembly

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**Abstract.** A plastome (chloroplast genome) mutant of tobacco, *lutescens-1*, displays abnormal degradation of the chloroplast-encoded polypeptides which form the core complex of photosystem II (PSII). Two nuclear-encoded proteins (present in polymorphic forms), which normally function in the water oxidation process of PSII, accumulate as larger size-class polypeptides in mutant thylakoid membranes. These accumulated proteins are intermediate in size between the full-length primary protein synthesized in the cytoplasm and the proteolytically processed mature polypeptides. Trypsin treatment of unstacked mutant thylakoids and of inside-out vesicle (PSII-enriched) preparations indicated that the intermediate size forms were correctly localized on the inner surface of the

thylakoid membrane, but not surface-exposed in the same way as the mature proteins. Only one of the intermediate size-class proteins could be extracted by salt washes.

We interpret these data to be consistent with the idea that the two imported proteins that function in the water oxidation step of photosynthesis and are localized in the loculus (the space within the thylakoid vesicles) undergo two-step processing. The second step in proteolytic processing may be related to transport through a second membrane (the first transport step through the chloroplast envelope having been completed); this step may be arrested in the mutant due to the absence of the PSII core complex.

THE biogenesis of the photosynthetic apparatus in higher plants requires an interplay between the nuclear and chloroplast genomes. The mechanism(s) by which nuclear-encoded, cytoplasmically synthesized proteins are targeted and properly sorted to their correct cellular location are not yet fully understood. In this process, proteins destined to be chloroplast membrane components must be transported across the chloroplast envelope, proteolytically processed to their mature size, and finally assembled with other subunits either in the stroma or thylakoid membranes (1).

Three nuclear-encoded polypeptides of 16, 23, and 33 kD are known to be involved in photosynthetic oxygen evolution (3). These proteins are associated with the pigmented core complex of photosystem II (PSII),<sup>1</sup> but are extrinsically bound at the inner thylakoid surface. They have been shown to be synthesized in the cytoplasm as larger, soluble precursors and posttranslationally imported into chloroplasts (30). The maturation of these proteins is particularly intriguing because their destination in the thylakoid lumen requires translocation across both the chloroplast envelope and thylakoid membranes.

1. *Abbreviations used in this paper:* Chl, chlorophyll; *lut-1*, *lutescens-1*; MES, 2[N-morpholino]-ethane sulfonic acid; OEC, oxygen evolving complex; PSII, photosystem II; WT, wild-type.

Our studies of a chloroplast-encoded photosynthetic tobacco mutant, *lutescens-1* (*lut-1*), afforded an opportunity to examine putative processing intermediates of two of the oxygen evolving complex (OEC) polypeptides (the 23- and 33-kD species). The phenotype of this mutant is a developmentally expressed loss of PSII activity. *Lut-1* PSII centers in immature leaves are functionally equivalent to those in wild-type (WT) chloroplasts; they have the normal capacity to carry out the process of oxygen evolution (7). During the course of normal plastid (leaf) ageing, however, *lut-1* chloroplasts undergo a specific and progressive loss of PSII polypeptides (8). Analyses of *lut-1* chloroplast-encoded PSII gene transcript levels and chloroplast protein synthesis indicated that reduced transcriptional and translational activities were not responsible for the physical depletion of PSII constituents. Instead the defect resulted from an inability of *lut-1* chloroplasts to retain adequate (WT) levels of PSII complexes, due to selective turnover of most of the protein components of the PSII core complex (Chia, C. P., J. L. Watson, and C. J. Arntzen, manuscript submitted for publication). This manuscript will describe the fate of the nuclear-encoded OEC proteins in membranes depleted of the PSII core complex.

## Materials and Methods

### Plant Material

WT and mutant *lut-1* tobacco (*Nicotiana tabacum* var L.C. from the Connecticut Agricultural Station) plants were grown as described previously (9). Mutant plants were maintained as variegated sectorial and/or periclinal chimeras, and pruned to force growth of mutant axillary buds which gave rise to completely mutant shoots suitable for chloroplast analysis. Thylakoids were isolated from 20–22-d (day one being leaf emergence from axillary buds) mutant and WT leaves.

### Isolation of Chloroplast Membranes (Thylakoids) and PSII Particles

Thylakoid membranes were prepared as described previously (9) except NaCl, MgCl<sub>2</sub>, and protease inhibitors were omitted from all buffers; the isolation medium was 400 mM sorbitol, 100 mM N-Tris-(hydroxymethyl)-methyl glycine (Tricine)-NaOH, pH 7.8, 5 mM sodium ascorbate, pH 7.8 and 0.3% (wt/vol) polyvinylpyrrolidone. Chloroplasts were washed twice in 10 mM Tricine-NaOH, pH 7.8 to ensure complete unstacking of the thylakoids. Chlorophyll (Chl) was determined by the method of MacKinney (21).

PSII particles were prepared as previously described (4) using the following modifications (23). Thylakoids were resuspended in 2(N-morpholino)-ethane sulfonic acid (MES) buffer (20 mM MES-NaOH, pH 6.5, 10 mM NaCl and 5 mM MgCl<sub>2</sub>) at 2 mg Chl/ml and incubated with Triton X-100 at a ratio of 1 to 20 (wt/wt; Chl to detergent) on ice, in the dark, with stirring for 30 min. The sample was then centrifuged at 40,000 g for 30 min. The resultant PSII pellet was resuspended in MES buffer and either used for trypsin experiments (described below) or treated with Tris/NaCl to remove the OEC polypeptides. The latter procedure involved dilution of the PSII particles to 1 mg Chl/ml, 1 M NaCl (with 4 M NaCl) and 50 mM Tris, pH 9.0, stirring for 15 min in the dark at 20°C, and centrifugation at 40,000 g for 20 min. The supernatant is referred to as the Tris/NaCl wash or extract, and contained the 16-, 23-, and 33-kD polypeptides comprising the OEC (17).

### Trypsin Treatments

Trypsin incubations were performed in the dark at room temperature with shaking on a rotary platform shaker (50 rpm). Thylakoid membranes were diluted in MES buffer to a final concentration of 250 µg Chl/ml. Trypsin (type III, from bovine pancreas, 12,000 U/mg protein; Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 37.5 µg/ml (final ratio 150 µg trypsin/mg Chl). PSII particles were subjected to trypsin digestion in an analogous manner except that the final trypsin concentration was 12.5 µg/ml, yielding a trypsin to Chl ratio of 50 µg trypsin/mg Chl. At times indicated in the figures, samples that were in the presence of trypsin were transferred into microfuge tubes and quickly centrifuged (13,000 g, 15 s). The supernatant was then removed by aspiration, 3× SDS sample buffer was added, and the sample was quickly vortexed and heated at 95°C for 1 min before SDS PAGE.

### Protein Detection by Immunoblotting

SDS PAGE was performed using the discontinuous buffer system of Laemmli (19) as described previously (9, 28). Gradient (10–17.5%) polyacrylamide SDS slab gels were run at a constant current of 15 mA. Molecular weight markers were: bovine serum albumin (BSA), 68 kD; ovalbumin, 45 kD; carbonic anhydrase, 29 kD; and cytochrome *c*, 12.4 kD.

Monospecific polyclonal antisera prepared against the purified 16-, 23-, and 33-kD spinach polypeptides, as previously described (2), were generously provided by Dr. C. Jansson, MSU/DOE Plant Research Laboratory, Michigan State University.

Proteins from denaturing SDS polyacrylamide gels were transferred to nitrocellulose sheets (0.45-µm pore size, Schleicher & Schuell, Inc., Keene, NH) for 6–10 h at 60 V with a Transphor Electrophoresis Cell (Hofer Scientific Instruments, San Francisco, CA). The transfer buffer was 25 mM Tris, 192 mM glycine, 20% vol/vol methanol, pH 8.3 (29). Nitrocellulose filters were quenched at room temperature for a minimum of 4 h in 20 mM Tris, 0.9% (wt/vol) NaCl, pH 7.4 (Tris-saline) and 3% (wt/vol) BSA. Blots were incubated with primary antibody (typically dilutions of 1/500 or 1/1,000 were used) in Tris-saline, 1% (wt/vol) BSA at 37°C for 1–3 h. Blots were washed three times, 15 min each, with Tris-saline, 0.1% (wt/vol) BSA,

0.1% (wt/vol) Triton X-100 (30 ml per 100 cm<sup>2</sup>) before addition of alkaline phosphatase conjugated to protein A (Sigma Chemical Co.; 10 U/100 ml) for 3 h at room temperature. Filters were washed three times, 15 min each, with 100 mM Tris, pH 7.5, 100 mM NaCl, 2 mM MgCl<sub>2</sub> and then washed twice, 10 min each, in alkaline phosphatase buffer (designated AP 9.5) containing 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub> at room temperature. The color detection of the immune complexes was performed using 0.33 mg nitro blue tetrazolium/ml of AP 9.5 and 16.7 mg 5-bromo-4-chloro-3-indoxyl phosphate per 333 µl dimethylformamide per 100 ml of AP 9.5 (20). After 15 min of color development in the dark, the reaction was stopped by washing filters in 10 mM Tris, pH 7.5, 1 mM EDTA for 15 min. Blots were stored in 10 mM Tris, pH 9.5, 5 mM EDTA before air drying.

### Isolation of Poly A<sup>+</sup>-RNA, In Vitro Translation, and Immunoprecipitation

Cytoplasmic RNA was isolated from WT tobacco and fractionated on a poly U Sepharose column following the procedure of Cashmore (6). Cell-free protein synthesis was performed with nuclease-treated rabbit reticulocyte lysates (Promega Biotec, Madison, WI) following supplier instructions (except volumes were increased five- to eightfold) using either [<sup>35</sup>S]methionine or [U-<sup>14</sup>C]leucine (Amersham Corp., Arlington Heights, IL).

Immunoprecipitation of translation products was carried out using modified procedures of Rochaix and Malnoe (25) and Schmidt et al. (27). After incubation of the translation mixture, 1 vol of NET buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA and 0.5% [wt/vol] Triton X-100) was added to 2 vol of lysate. To this mixture, one-tenth volume of preimmune serum was added and the solution was shaken on a rotary platform shaker (50 rpm) for 1 h at room temperature. One-tenth volume of a slurry of protein A-Sepharose beads (Pharmacia Fine Chemicals, Inc., Piscataway, NJ), washed twice in 100 mM phosphate buffer, pH 7.0 and resuspended (125 mg/ml) in 25 mM Tris-HCl, pH 7.5, was added, and the mixture was shaken at room temperature for 1 h. Beads were separated from the mixture by centrifuging samples for 1 min in a microfuge and discarded. One-twentieth volume of immune serum was added to the mixture which was then shaken either overnight at 4°C or at 37°C for 4 h. A volume of fresh protein A-Sepharose beads equal to the immune serum volume was added and the mixture was again shaken either overnight at 4°C or at 37°C for 4 h before beads were collected by centrifugation. Pelleted beads were washed twice with 500 µl NET and then twice with 750 µl of wash buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.5 and 1% [vol/vol] 2-mercaptoethanol). Elution of the immune complex was achieved by boiling beads for 1 min in 40 µl of 3× SDS sample buffer. Samples were analyzed by SDS PAGE.

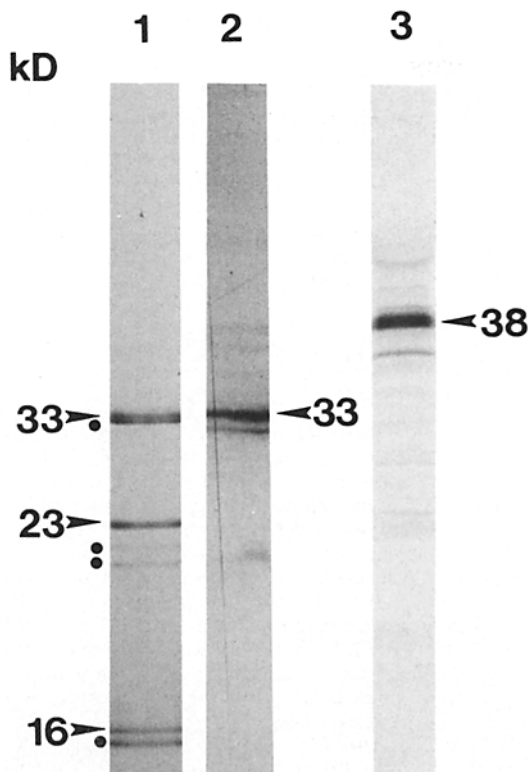
## Results

### Polypeptides of the OEC

The OEC has been biochemically characterized in several plant species, but most extensively in spinach where it is comprised of three proteins of 16, 23, and 33 kD. The procedures developed to isolate the OEC from spinach are generally applicable for chloroplasts of other plant species, even though the apparent molecular weights of the isolated proteins sometimes vary (in maize, for example, the three proteins are 14, 25, and 32 kD; reference 5).

Preparation of the OEC from *N. tabacum* by the high pH-high salt wash procedure (1, 17) resulted in a preparation containing seven polypeptides (Fig. 1, lane 1), rather than three as in spinach. These seven proteins fell in three size-classes: a very closely spaced doublet of ~33 kD, three proteins of 21, 22, and 23 kD, and a doublet of 15.5 and 16 kD. Polyclonal antibodies prepared against the spinach OEC reacted with this complex of seven proteins.

*N. tabacum* is an allotetraploid (2n = 48) which is the result of a hybridization of *N. sylvestris* (2n = 24) with *N. tomentosiformis* (2n = 24) (15). For comparison to *N. tabacum*, the OEC was prepared from *N. sylvestris* and *N. tomen-*



**Figure 1.** Mature and primary product size of OEC polypeptides. Lane 1, Tris/NaCl extract containing WT OEC polypeptides separated by SDS PAGE and stained with Coomassie Blue. The multiple bands for each species (●) were polymorphic forms (see Results). Lanes 2 and 3, autoradiograms of immunoprecipitated primary products synthesized in vitro using poly A<sup>+</sup>-RNA, isolated from WT tobacco leaves, in a rabbit reticulocyte cell-free translation system. Lane 2, anti-23-kD serum immunoprecipitated a major band at 33 kD (arrow) and a minor band at 32 kD; lane 3, anti-33-kD serum immunoprecipitated a major band at 38 kD (arrow) and a minor band at 35 kD.

*tosiformis* (Duesing, J. H., P. Yang, and C. J. Arntzen, unpublished data). Each parental species contained only three proteins in the OEC complex when these were compared to the *N. tabacum* OEC by SDS PAGE; each member of the "16" and "33" kD doublets of *N. tabacum* corresponded directly to a similarly sized protein in the parental strains. We interpret this polymorphism to indicate that the *N. sylvestris* and *N. tomentosiformis* genomes each contained an allele for these polypeptides that was expressed in *N. tabacum*. These gene products or polymorphic forms were of slightly different mature size. In the case of the 23-kD size-class, two of the *N. tabacum* OEC polypeptides corresponded to proteins isolated from the parental lines. Presumably these also represented products of separate alleles in the combined genome. The third polypeptide in this size class was also recognized by an antibody to the spinach 23-kD protein. We suggest this species is either a degradation product or an altered processing product of one of the two 23-kD size-class proteins in the OEC of *N. tabacum*.

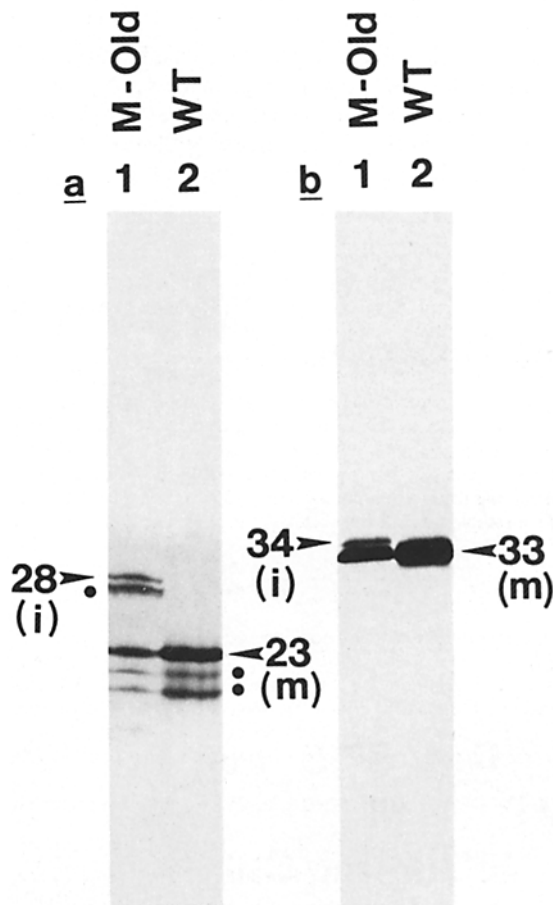
In summary, *N. tabacum* contains seven polypeptides in its OEC. These fall into three size classes (~16, 23, and 33 kD), each member of which reacts with monospecific antisera reactive against the spinach 16-, 23-, or 33-kD proteins. For

the remainder of this paper we shall consider the polymorphic forms within each size class to be related (since all members of a group showed parallel behavior), and simply refer to the "16", "23", or "33"-kD proteins.

### Primary Product Size

In comparison to chloroplasts from WT tobacco, chloroplasts from fully expanded leaves of *lut-1* are depleted in nuclear- and chloroplast-encoded PSII polypeptides (7). In examining the levels of the 23- and 33-kD OEC proteins by immunoblotting, higher molecular weight species at 28 (a doublet probably due to polymorphism; Fig. 2 a) and 34 kD (Fig. 2 b), respectively, were observed to accumulate in thylakoid membranes from these mutant chloroplasts concomitant with a reduction in the level of the mature protein species.

Both the 23- and 33-kD polypeptides of the OEC are synthesized in the cytoplasm as larger (by 10 and 6 kD, respectively) precursor molecules in spinach (30). To verify that the tobacco cytosolic precursors were also made as larger size polypeptides, poly A<sup>+</sup>-RNA was isolated from WT tobacco and translated in a rabbit reticulocyte cell-free system. The synthesis of the precursors to the 23- and 33-kD



**Figure 2.** Protein blots of *lut-1* and WT thylakoid membranes probed with antisera prepared against the mature (a) 23-kD and (b) 33-kD polypeptides of spinach (2). Higher molecular weight forms, at 28 and 34 kD, respectively (henceforth termed intermediates [i]), were observed to accumulate in mutant thylakoids while the mature (m) forms were lost. ●, putative polymorphic forms (see Results).

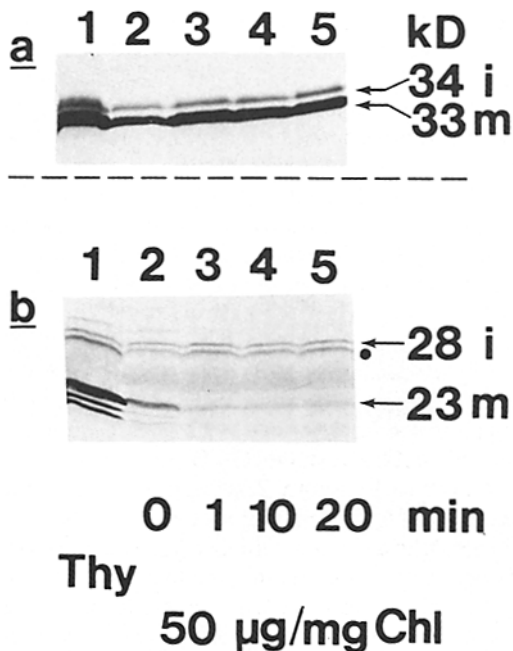
proteins was checked by immunoprecipitation with mono-specific antisera (Fig. 1). The primary translation product reacting with the 23-kD antisera migrated at 33 kD (Fig. 1, lane 2). A doublet was again observed for this polypeptide. The two species are possibly derived from mRNAs from each of the two different parental alleles, although proteolysis and/or early termination of translation cannot be ruled out.

The primary translation product reacting with the 33-kD antisera migrated at 38 kD. In addition, a smaller protein at 35 kD was observed (Fig. 1, lane 3). The 35-kD labeled species was larger than the membrane-bound precursor observed on immunoblots (see Fig. 2) and may have been the result of early termination of translation in the rabbit reticulocyte system.

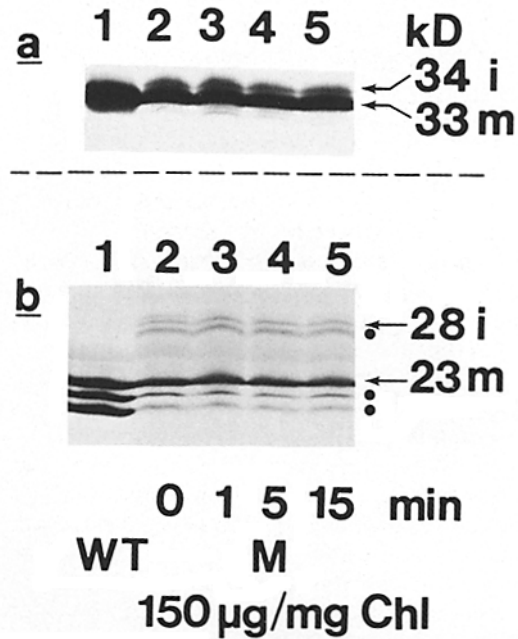
These results show that the membrane protein preparations of *lut-1* thylakoids, in which the 28- and 34-kD polypeptides were detected, were not contaminated with cytoplasmically synthesized precursor proteins. The 28- and 34-kD polypeptides were considered to be intermediate proteins because they were intermediate in size between the primary protein products and the mature, processed products. Investigations to determine the location of these intermediates are described below.

### Topology

Several tests were carried out to determine whether the intermediate forms of the OEC proteins were in their proper location on the luminal side of thylakoid membranes. The first approach was to proteolytically digest well-washed, unstacked mutant thylakoid membranes and look for removal



**Figure 3.** Protein blots of WT and trypsin-treated ( $150 \mu\text{g}$  trypsin/mg Chl; incubation times are indicated below the figure) unstacked mutant thylakoid membranes (*M*) probed with antisera prepared against the (a) a 33-kD or (b) 23-kD polypeptides. Arrows indicate the mature (*m*) and intermediate (*i*) forms of the two OEC polypeptides. ●, polymorphic forms. Their trypsin insensitivity indicated that they were not exposed to the stromal phase.



**Figure 4.** Protein blots of *lut-1* thylakoids (*THY*) and trypsin-treated ( $50 \mu\text{g}$  trypsin/mg Chl; incubation times are indicated below the figure) inside-out vesicles (PSII-enriched preparations) derived from mutant thylakoids (see Materials and Methods) probed with antisera reactive against the (a) 33-kD or (b) 23-kD polypeptides. Arrows indicate the mature (*m*) and intermediate (*i*) forms of the two OEC polypeptides. ●, polymorphic form of the intermediate 28-kD polypeptide. Under these conditions, neither the intermediate 34-kD nor mature 33-kD species was digested. Higher trypsin to Chl ratios did achieve proteolysis of both forms. The mature 23-kD polypeptide, but not the intermediate 28-kD form was removed by this trypsin treatment.

of the higher molecular weight species. Under the conditions used, surface-exposed membrane proteins such as the LHC-II (28) were partially digested (data not presented). Under the trypsin digestion conditions used ( $150 \mu\text{g}$  trypsin/mg Chl) neither the 34- nor the 28-kD intermediate forms were altered in size (Fig. 3, a and b). Higher trypsin to Chl ratios were effective in cleaving the intermediates, but these conditions also digested the mature forms, indicating penetration of the protease through the membranes (data not shown). From these experiments, we concluded that the intermediate forms of the OEC were either inserted into the thylakoid membranes or translocated across them, and not merely adhering to the thylakoid surface.

These experiments on intact membranes, which are right-side-out, were complemented by analogous experiments with detergent-prepared PSII particles. These particles are grana-enriched thylakoid fragments which are inside-out vesicles, and consist primarily of unsealed pairs of membranes stabilized at the edges by detergent (10). In these PSII preparations the inner thylakoid surface is exposed to added proteolytic enzymes. The PSII particles prepared from *lut-1* membranes were subjected to a time course of trypsin digestion ( $50 \mu\text{g}$  trypsin/mg Chl). The immunoblots are shown in Fig. 4. Under the conditions used, neither the mature 33-kD nor intermediate 34-kD forms were removed by this trypsin treatment (Fig. 4 a). The mature 23-kD polypeptide was

quickly digested away, whereas the intermediate form was not (Fig. 4 *b*). At higher trypsin to Chl ratios, both intermediate and mature forms of the 23- and 33-kD proteins could be removed, indicating that all species were accessible to the protease although penetration of the enzyme cannot be ruled out (data not shown). An experiment identical to that shown in Fig. 4 was performed with WT thylakoids. The results were the same: the mature 23-kD protein was digested more rapidly than the mature 33 (data not shown).

These data are consistent with the postulated topography of the polypeptides comprising the OEC presented in reference 3 and the models proposed by others (14). The 33-kD protein is closely associated with the core complex of PSII, whereas the 23-kD protein is more extrinsically located with greater exposure to the lumen space. The 23-kD protein is therefore more susceptible than the 33-kD protein to trypsin cleavage. In contrast to the mature 23-kD protein, the 28-kD intermediate observed in *lut-1* membranes was partially protected from proteolytic digestion, which suggests that the latter form was not in its proper membrane location (Fig. 4). Conceivably, it was at least partially sequestered in the thylakoid membrane itself, rendering it inaccessible to proteolytic degradation. There was no differential degradation of the 33- and 34-kD forms under the conditions described here or under harsher trypsin treatments (data not shown).

A third test devised to examine the position of the intermediate-size OEC proteins was to remove the OEC polypeptides from PSII particles with an alkaline pH-high salt (NaCl) wash. In previous studies with spinach PSII preparations, it was shown that the 16-, 23-, and 33-kD proteins may

be extracted from inside-out vesicles with Tris-HCl, pH 9.3 (18), whereas the 23- and 16-kD proteins but not the 33-kD species are removed with a 1 M NaCl wash (1). Inside-out vesicles were prepared from mutant chloroplasts and subjected to the OEC extraction conditions, followed by immunoblotting analysis. The results are shown in Fig. 5. In *a*, protein blots were probed with anti-33-kD sera; in *b*, blots were probed with anti-23-kD sera. Mutant PSII particles prepared from *lut-1* thylakoids are shown in lane 3. The yield of PSII was low but both the mature 23- and its 28-kD intermediate were present in this preparation. Lane 4 is the PSII particle depleted of the mature OEC polypeptides; lane 5 is the Tris/NaCl extract. It can be seen that the 28-kD intermediate species was not extracted by the alkaline pH-high salt wash. It remained in the depleted PSII fraction. The alkaline pH-high salt wash contained the mature 23-, but lacked the 28-kD form. In contrast, both the 34-kD intermediate and the mature 33 were extracted from the mutant PSII complex (Fig. 5 *b*, lane 5). Although the extraction was incomplete, it is clear that the 34-kD intermediate responded to extraction in a fashion like that of the 33-kD protein and was removed under the conditions that removed the mature form.

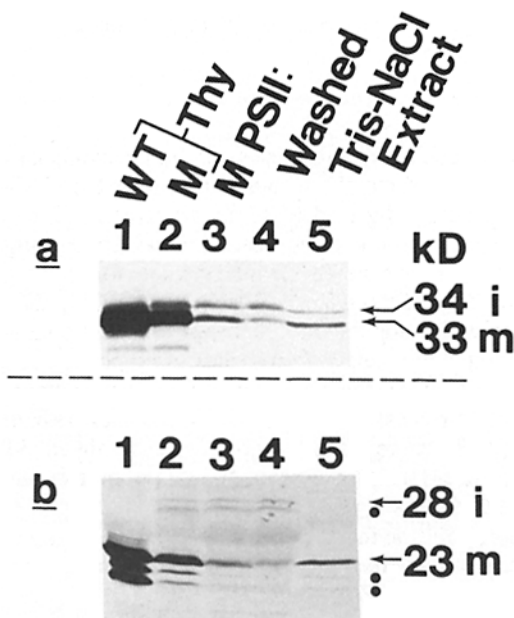
## Discussion

Extensive previous research has shown that three polypeptides of 16, 23, and 33 kD are involved in oxygen evolution reactions in spinach chloroplasts (3). These proteins are extrinsically localized on the inner thylakoid surface (2) and can be extracted from inside-out thylakoid vesicles by high pH-high salt washes (17, 18).

The results obtained in this study show that the OEC of *Nicotiana tabacum* chloroplasts contains seven polypeptide species that fall in three size classes near 16, 23, and 33 kD (Fig. 1). The use of monospecific antibodies prepared against each of the individual spinach proteins (2) provided evidence that each spinach polypeptide corresponds to a doublet or triplet set of proteins in the tobacco chloroplast. We conclude that two or more polymorphic forms of each OEC protein are present in tobacco due to its genetic composition (a tetraploid nuclear genome; see Results section).

Chloroplasts in early stages of leaf expansion in tobacco plastome mutant, *lut-1*, contain an active PSII (7), including OEC polypeptides of the mature size classes (Fig. 1). In fully expanded leaves, however, the PSII core complex proteins are absent from *lut-1* thylakoids (8), and monospecific antibodies to either the 23- or 33-kD proteins recognize larger size-class polypeptides of ~28 and 34 kD, respectively (Fig. 2). The sizes of the primary protein products, which were detected by immunoprecipitation after their synthesis in cell-free translation assays, were 33 kD, 10 kD larger than the mature 23-kD protein, and 38 kD, 5 kD larger than the mature 33-kD protein; the 28- and 34-kD polypeptides detected in the mutant membranes are therefore "intermediate-size proteins".

The 28- and 34-kD intermediate-size class OEC polypeptides were thylakoid associated and not subject to digestion by trypsin addition to right-side-out thylakoids (Fig. 3). The 34-kD protein was present and subsequently removed from the inner thylakoid surface with a high pH-high salt wash in tandem with the 33-kD mature form (Fig. 5). In contrast, the



**Figure 5.** Protein blots probed with antisera reactive against the (a) 33-kD and (b) 23-kD polypeptides. Lanes are: (1) WT thylakoids, (2) *lut-1* thylakoids, (3) a *lut-1* PSII-enriched preparation, (4) a *lut-1* PSII-enriched preparation remaining after extraction with Tris/NaCl, and (5) a Tris/NaCl extract of a *lut-1* PSII-enriched preparation. The intermediate (i) 34 kD, mature (m) 33- and 23-kD forms were extracted by the Tris/NaCl treatment (lane 5). The intermediate 28-kD species was not extracted and remained with the membrane fraction (lane 4). ●, polymorphic forms of the mature 23- and intermediate 28-kD polypeptides.

28-kD species were removed neither by trypsin (Fig. 4 b) nor by a Tris/NaCl wash (Fig. 5 b) from inside-out vesicles (PSII particles), unlike the mature 23-kD form. This indicated that the attachment of the 28-kD intermediate form to membranes was more hydrophobic in nature than the association of the mature 23-kD species to thylakoids, and that the 28-kD intermediate possibly lacked surface-exposed domains.

We have previously concluded that PSII core complexes are prematurely removed from *lut-1* mutant thylakoids due to an accelerated turnover of the core polypeptides (Chia, C. P., J. L. Watson, and C. J. Arntzen, manuscript submitted for publication). The current study has shown that intermediate-size class OEC proteins accumulate under these conditions. We now suggest that this accumulation is due to the inability of the thylakoid system to complete the last step in a two-step processing of the OEC proteins due to an absence of their membrane-binding site (the PSII core complex). That is, the putative intermediates are not assembled into PSII complexes, although our data indicate that they are properly localized close to the inner thylakoid surface.

In the mutant membranes that accumulate the 28- and 34-kD intermediates, other protein processing steps appear to occur normally. The third nuclear-encoded (16 kD) OEC polypeptide for which the primary translation product is ~26 kD (30), and the 32-kD chloroplast-encoded protein which is synthesized as a 34.5-kD primary product (16), are both processed normally; we did not observe higher molecular weight forms of these proteins by immunoblotting analysis (data not shown). Protein blots were also probed with a monoclonal antibody against the major nuclear-encoded light-harvesting Chl apoprotein. No higher molecular weight forms were observed (data not shown). The 23- and 33-kD polypeptides of the OEC appeared to be the only species of the PSII complex to have accumulated as intermediate forms in *lut-1* thylakoids.

There are now several lines of evidence for two-step processing of proteins imported into organelles. Two-step processing of yeast mitochondrial proteins was delineated when required co-factors were absent or when a needed transmembrane potential was artificially collapsed in yeast mitochondria (12, 13). Processing intermediates have been shown for the small subunit of ribulose-bisphosphate carboxylase (Rubisco) in pea (22, 24) and for the L-18 chloroplast ribosomal protein from *Chlamydomonas* (26). The Rubisco small subunit intermediate was observed in a heterologous system (22) and in vitro (24); the L-18 intermediate was observed in vivo (26).

In conclusion, we have provided evidence consistent with the idea that processing of two OEC polypeptides, both of which normally reside in the chloroplast lumen, occurs in two steps. One of these (the 28-kD intermediate form of the 23-kD mature protein) does not reach its proper site of localization. We hypothesize that the final stage of processing of these proteins requires their site of binding—the PSII core complex—and perhaps the integration of co-factors such as manganese, chloride, and/or calcium ions which are known to be involved in the water oxidation process (14).

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