Plant Physiol. (1996) 111: 1329–1338

Analysis of the Import of Carboxyl-Terminal Truncations of the 23-Kilodalton Subunit of the Oxygen-Evolving Complex Suggests That Its Structure Is an Important Determinant for Thylakoid Transport[1]

Robin A. Roffey and Steven M. Theg*

Section of Plant Biology, University of California, Davis, California 95616

A series of deletions from the carboxyl terminus of the 23-kD subunit of the photosynthetic oxygen-evolving complex OE23 revealed that these truncations result in various degrees of inhibition of translocation across thylakoid membranes and their subsequent assembly to the oxygen-evolving complex. Import of in vitro translated precursors across the chloroplast envelopes was not inhibited by these truncations. Time-course studies of the import of truncated OE23 precursors into intact chloroplasts revealed that the stromal intermediate was subsequently translocated into the thylakoid lumen, where it was processed to a smaller size and rapidly degraded. In contrast to the full-length OE23 intermediate, the truncated intermediate forms that accumulated in the stroma as a result of de-energization of thylakoid membranes could be found associated with the membrane rather than free in the stroma. Protease digestion experiments revealed that the deletions evidently altered the folded conformation of the protein. These results suggest that the carboxyl-terminal portion of the OE23 precursor is important for the maintenance of an optimal structure for import into thylakoids, implying that the efficient translocation of OE23 requires the protein to be correctly folded. In addition, the rapid degradation of the truncated forms of the processed OE23 within the lumen indicates that a protease (or proteases) active in the lumen can recognize and remove misfolded polypeptides.

Within the chloroplast, the multisubunit PSII protein complex is the site of catalysis for the conversion of water to molecular oxygen as a result of photosynthetic electron transfer across thylakoid membranes. The minimal PSII preparation from eukaryotic photosynthetic membranes that is able to catalyze oxygen evolution consists of the 32- and 34-kD integral protein subunits known as D1 and D2, which make up the core of the reaction center; the α and β subunits of Cyt b_{559}; the core antenna components CP43 and CP47; and several other small polypeptides (reviewed by Debus, 1992; Vermaas, 1993). Most oxygen-evolving preparations also contain a 33-kD extrinsic protein (OE33), although its presence is not strictly required for this activity. Two additional extrinsic protein components of 23 and 17 kD (OE23 and OE17) also are associated with a more intact oxygen-evolving membrane preparation. The extrinsic subunits of the OEC, OE33, OE23, and OE17 are tightly bound to the lumen-exposed domains of the PSII complex. Loss of the OE23 and OE17 proteins has been correlated with lowered rates of oxygen evolution, but because depleted membranes retain the ability to catalyze water oxidation, these polypeptides are considered to have structural and regulatory roles, as opposed to catalytic functions, in oxygen evolution (Vermaas, 1993).

The assembly of the OEC is of interest not only for its implications in mechanistic and regulatory aspects of oxygen evolution, but also because the multimeric PSII-OEC is formed with subunits encoded by both chloroplast and nuclear genes. Although most of the integral PSII subunits are synthesized in the chloroplast, the nuclear-encoded extrinsic subunits are synthesized on cytoplasmic ribosomes and must be translocated across the chloroplast envelope membranes and thylakoid membranes before assembly into the OEC in the lumen (reviewed by Keegstra et al., 1989; Theg and Scott, 1993).

Most nuclear-encoded plastid proteins, including the OEC extrinsic subunits, are targeted to the thylakoid lumen by a cleavable, amino-terminal presequence known as a transit peptide. The majority of transit peptides directing polypeptides to the thylakoid lumen have a bipartite structure, with a chloroplast-targeting domain at the amino terminus and a lumenal targeting domain at the carboxyl end of the transit sequence. The transit peptide is removed by specific proteases upon translocation of the protein (Theg and Scott, 1993).

Experimentally, in vitro transcribed and translated protein precursors can be imported into isolated chloroplasts by incubation under illumination or in the presence of ATP (Theg et al., 1989). Information contained in the transit peptide directs these polypeptides to the correct compartment within the chloroplast, where the targeting sequence is removed, resulting in the mature-sized protein. Chloroplast subfractionation experiments have revealed that the extrinsic OEC subunits reside in two distinct locations: on

[1] This work was supported by grant no. DE–FG03–93ER20118 from the U.S. Department of Energy to S.M.T.

* Corresponding author; e-mail smtheg@ucdavis.edu; fax 1–916–752–5410.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; OEC, oxygen-evolving complex; OE33, OE23, and OE17, the 33-, 23-, and 17-kD subunits of the oxygen-evolving complex, respectively; OE23–1, A through E, parent and C-terminal truncations of OE23 as described in Figure 1.

the membrane assembled into the OEC and in a stable soluble pool within the lumen (Ettinger and Theg, 1991). Experiments with radiolabeled OEC subunit precursors indicate that newly imported subunits take up residence in these same two locations (A. Hashimoto, unpublished data). These two populations of newly imported precursors can be distinguished empirically by minimal solubilization of the thylakoids with low concentrations of detergent followed by centrifugation; assembled proteins pellet with the thylakoid membranes and the lumen pool residents remain in the supernatant.

Whereas the chloroplast envelope membranes are capable of importing the majority of polypeptides by a single, well-characterized pathway, translocation into or across the thylakoid membranes occurs by at least one of four distinct, polypeptide-specific mechanisms. In one pathway, proteins appear to insert into the membrane spontaneously by virtue of their hydrophobicity (Michl et al., 1994). A second pathway is utilized by the major light-harvesting chlorophyll-binding protein of PSII and uses a homolog of the signal recognition particle involved in ER protein translocation (Li et al., 1995). A third pathway is followed by a subset of proteins represented by OE33 and plastocyanin and makes use of at least one homolog of the bacterial Sec-dependent secretion pathway (Nakai et al., 1993, 1994; Yuan et al., 1994). The fourth pathway, which is utilized by proteins such as OE23 and OE17, harvests the energy contained within the energy-rich transmembrane pH gradient to drive proteins across the thylakoid membrane into the lumen. This is the only known polypeptide translocation system that does not require the input of energy from the hydrolysis of a high-energy phosphodiester bond to power protein transport. The components of the translocation machinery functioning in this pathway are not known and may be without precedent in other protein-transporting systems (Cline et al., 1992; Robinson et al., 1993).

The OE23 and OE17 subunits are most likely to bind to the PSII complex by means of electrostatic attractions, since they are easily removed by 1.0 M NaCl. The OE33 subunit, however, is more tightly associated with the integral complex and must be removed with urea or high concentrations of $CaCl_2$. Depletion and reconstitution experiments using detergent-isolated PSII membranes or inside-out thylakoids have provided some information about how the OEC subunits are assembled (reviewed by Ghanotakis and Yocum, 1990). Rebinding experiments have shown that the presence of the OE33 subunit facilitates the binding of the OE23 protein, although OE23 associates with the complex in the absence of bound OE33 (Miyao et al., 1988; Miyao and Murata, 1989). The binding of OE33 to the integral PSII components appears to result in a conformational change in the complex that may either enhance an intrinsic OE23-binding site or create a binding site on OE33 itself (Miyao and Murata, 1989). Although these subunits do not associate in solution, additional evidence for their interaction comes from cross-linking studies in intact complexes (reviewed by Ghanotakis and Yocum, 1990). Although it is unclear whether the OE23 binds directly to the intrinsic polypeptides or to a site on the OE33 subunit, there is some evidence for the specific association of OE23 and the intrinsic CP43 based on the correlated presence of these subunits in specific regions of the thylakoids in mutants of *Chlamydomonas reinhardtii* (de Vitry et al., 1989). Cross-linked products of the OE23 and OE17 proteins have also been observed, and assembly of both the OE33 and OE23 proteins onto PSII complexes is required for the functional binding of OE17 (Miyao and Murata, 1989).

During the course of investigations of the binding of OE23 to the OEC, we constructed a series of truncated OE23 precursor cDNA clones for use in import and assembly assays of in vitro translated proteins. Upon examining these constructs we made two surprising observations. First, even though the amino-terminal regions (including the transit peptides) of the truncated precursors were completely intact and functioned for efficient transport across the chloroplast envelope membranes, these shortened proteins were transported across the thylakoid membranes very poorly. Second, in marked contrast to the wild-type OE23, those truncated polypeptides that were translocated into the thylakoid lumen were not assembled into the OEC and were subjected to rapid proteolysis.

MATERIALS AND METHODS

Chloroplast and Thylakoid Preparation

Intact chloroplasts were isolated as described previously by Ettinger and Theg (1991) from 10- to 14-d-old pea (*Pisum sativum* cv Progress 9) seedlings and kept on ice in import buffer (Hepes/KOH, pH 8.0, and 0.33 M sorbitol) until use. Chloroplast lysates were prepared by osmotic lysis in 10 mM Mes/KOH, pH 6.5, and 5 mM $MgCl_2$ on ice for 10 min. Thylakoids were separated from the soluble stroma by centrifugation at 4000*g* for 5 min and resuspended in import buffer containing 5 mM $MgCl_2$.

Preparation of Radiolabeled Precursors

A slightly (and for this study, inconsequentially) modified pea cDNA clone (Wales et al., 1989) encoding the precursor of OE23 (prOE23–1) served as the parent plasmid from which the truncated proteins used for these experiments were derived. A series of truncations of the carboxyl end of the polypeptide were constructed utilizing existing restriction sites (*Fok*I, *Sac*I, and *Mbo*I) in prOE23–1. The OE23–1A and OE23–1B truncations were generated by PCR-directed mutagenesis to substitute stop codons (TAG) at sites 30 and 69 bases, respectively, from the 3′ end of prOE23–1.

RNA for in vitro translation of full-length and truncated prOE23 was transcribed from linearized plasmids containing the full-length and truncated prOE23–1 cDNA clones using the SP6 RNA polymerase (Promega). Translation of the mRNA was performed in a cell-free wheat germ extract containing [^{3}H]Leu (NEN-Dupont). The translation reactions were terminated by the addition of an excess of nonradiolabeled Leu.

Chloroplast Import Assays

Import of the OE23 precursor proteins into isolated chloroplasts was carried out essentially as described by Theg et al. (1989). Import assays were initiated by the addition of isolated chloroplasts (0.33 mg chlorophyll/mL) to import medium (330 mM sorbitol, 50 mM K-Hepes, pH 7.5, 5 mM $MgCl_2$, and 3 mM ATP) containing in vitro synthesized, ^3H-labeled precursor protein. To block translocation across the thylakoid membrane in some assays ethanolic stock solutions of the ionophores nigericin and valinomycin or CCCP were added to the import reaction mixture at the concentrations indicated in the figure legends. Following import under illumination or in the dark for the time indicated in the figure legends, chloroplasts were re-isolated by centrifugation through 40% Percoll (Pharmacia) and subfractionated into soluble and membrane components. For import time-course assays, reactions were initiated in the light by the addition of chloroplasts, and at the indicated times aliquots were removed and centrifuged through silicon oil into 1.5 M perchloric acid (Theg et al., 1989). All import samples were recovered in buffer containing SDS and β-mercaptoethanol and subjected to SDS-PAGE and fluorography.

Subchloroplast Fractionation

Chloroplasts were re-isolated by centrifugation through 40% Percoll and subfractionated into soluble and membrane components. Following osmotic lysis and centrifugation of the samples, the chloroplast envelope membranes and intact thylakoids were recovered in the pellet, and the stroma was recovered in the supernatant. Further treatment of the membrane pellet with 0.1% Triton X-100 (Sigma), followed by centrifugation at 100,000*g* for 8 min, yielded membranes in the pellet and the thylakoid lumen content in the soluble phase.

Protease Sensitivity Assays

The in vitro translated precursors were incubated for 20 min on ice with the indicated concentration of protease. Reactions were terminated by the addition of protease inhibitors as stated in the legend to Figure 8, and samples were analyzed directly by SDS-PAGE and fluorography.

RESULTS

Modifications of the OE23 Precursor

To facilitate future experiments we generated an Asp → Met substitution immediately preceding the coding sequence of the mature protein by PCR-directed mutagenesis of the pea cDNA clone (p23SP6) (Fig. 1). The in vitro translated precursor product of the modified OE23 clone, prOE23–1, behaved identically to the wild-type precursor with respect to chloroplast import, internal targeting, and assembly onto the thylakoid membrane (Fig. 2; A. Hashimoto, unpublished data). Therefore, the OE23–1-modified cDNA was used to generate the truncated precursors described below.

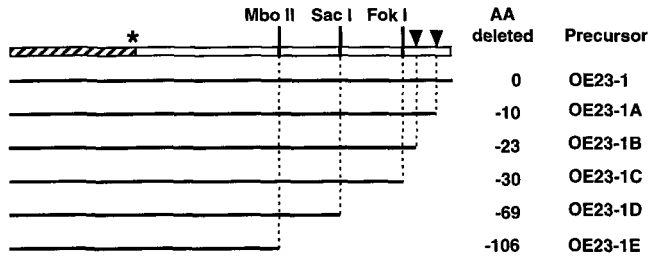

Figure 1. Summary of the OE23–1 precursors used in import studies. The open box and the hatched box represent the cDNA clone sequence for the mature portion and the transit peptide regions, respectively, of the OE23 precursor. The arrowheads indicate the placement of stop codons to generate truncated precursors; the asterisk (*) indicates the substitution of a Met codon at the start of the mature region; the restriction sites used to generate truncated precursors are indicated. The solid lines represent the relative length of the precursors following truncation by the number of amino acids indicated on the right.

Since there is no structural information available for the extrinsic subunits of PSII, it was necessary to take a systematic approach to the mutational analysis of the OE23 polypeptide. Although very little data exist concerning the binding properties of specific OE23 protein domains, reconstitution studies of native and protease-treated OE23 have suggested that the amino-terminal portion of the mature protein is not essential for functional binding to the PSII complex (Miyao et al., 1988). Using this information we initially generated a series of truncations from the carboxyl end of the polypeptide utilizing existing unique restriction sites in the cDNA. The restriction enzymes used and the resulting truncated OE23 precursors are shown in the diagram in Figure 1.

Import of the Truncated OE23 Precursors into Chloroplasts

The truncated clones were transcribed and translated in vitro and assessed for their ability to assemble into the OEC. After 30 min of import in the light, no mature truncated OE23 protein could be observed in the thylakoid fraction following re-isolation and fractionation of chloroplasts (data not shown). Similar results were obtained following import into isolated thylakoids (data not shown). Since the import efficiency of these truncated precursors was too low for in organello assembly assays, it became important to determine if the lack of a translation stop codon had caused the newly made polypeptides to remain associated with ribosomes, rendering them incompetent for translocation across the chloroplast membranes. To examine this question, and also to determine the extent to which the carboxyl end of the OE23 protein is important for its proper targeting, PCR-directed mutagenesis was used to insert stop codons at the carboxyl terminus of the prOE23 precursor. These truncations eliminated only 10 or 23 amino acids from the end of the OE23 polypeptide (Figs. 1 and 2).

After 30 min of import into chloroplasts and the subsequent subchloroplast fractionation, the OE23–1A and

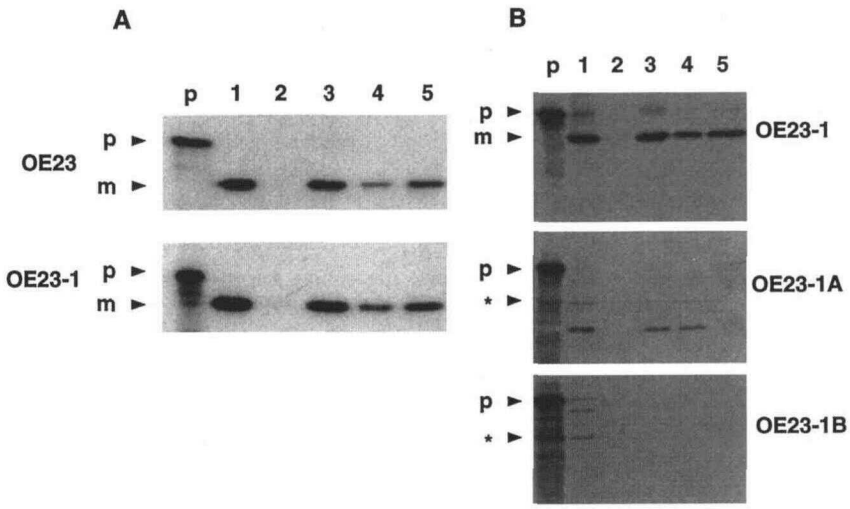

Figure 2. Assembly of OE23 and modified OE23–1 after import of the precursors into chloroplasts. A, prOE23–1 is imported into isolated chloroplasts and properly assembled in the thylakoid lumen. B, The truncated prOE23–1 polypeptides are not efficiently imported into chloroplasts or assembled in the thylakoid lumen. Import reactions were initiated by the addition of isolated chloroplasts (0.33 mg chlorophyll/mL) to the import medium (330 mM sorbitol, 50 mM K-Hepes, pH 7.5, 5 mM $MgCl_2$, and 3 mM ATP) containing in vitro synthesized, ^{3}H-labeled precursor protein. Following 30 min of import in the light, chloroplasts were re-isolated by centrifugation through 40% Percoll and subfractionated into soluble and membrane components. Radiolabeled proteins were visualized by SDS-PAGE and fluorography. Lane p, Precursor; lane 1, intact chloroplasts; lane 2, soluble fraction following osmotic lysis (chloroplast stroma); lane 3, membrane fraction (envelope membranes and thylakoids); lane 4, soluble (thylakoid lumen) fraction; and lane 5, membrane fraction (envelope and thylakoid membranes). Samples in lanes 4 and 5 were derived from the sample in lane 3 by detergent (0.1% Triton X-100) treatment and centrifugation at 100,000*g* for 8 min. p, Precursor OE23; m, mature OE23; asterisk, low-molecular-weight OE23.

OE23–1B truncated precursors appeared to be inefficiently imported into the chloroplasts (Fig. 2B), a finding similar to those obtained with the restriction site-generated truncations. The small amount of low-molecular-weight protein that did appear in the thylakoid fraction did not associate with the membrane but was present in the soluble fraction following detergent (Triton X-100) release of lumen contents (Fig. 2B). In addition, these protein fragments migrated in SDS-PAGE as lower molecular weight species than would be expected for the −10 and −23 amino acid truncations. These data suggested that, despite the presence of proper translation termination codons, and with relatively few amino acids removed from the carboxyl end of the precursor, the import efficiency of these truncated OE23 precursors was greatly impaired.

Kinetics of Chloroplast Import

It was impossible to determine from the initial import studies whether the transport of the truncated precursors was blocked at the chloroplast envelope or at some later step, since neither mature-sized proteins nor stromal intermediates were detected. For instance, if the truncated precursors were transported into the chloroplast and rapidly degraded, the internalized protein might escape detection in a 30-min import assay. To examine this possibility, time-course assays were designed to monitor chloroplast import of full-length and truncated OE23 as a function of time. The import conditions were identical to those in the previous assays, but aliquots of the import mixture were removed at specific times and centrifuged through silicon oil into perchloric acid to terminate the transport reaction. As shown in Figure 3, the stromal intermediate derived from the full-length OE23–1 precursor was first observed at 2 min, reached its maximal level at 5 min, and had almost entirely disappeared by the 10-min point. The disappearance of the stromal intermediate was paralleled by the appearance of the mature OE23, as expected from their precursor-product relationship.

Import time courses for each of the truncated OE23 precursors revealed a transient stromal intermediate form, indicating that the precursors were indeed translocated across the chloroplast envelope (Fig. 3). The stromal intermediate of every OE23 construct tested appeared within 2 min, suggesting that translocation across the chloroplast envelope occurred at rates similar to that of the full-length OE23 precursor. Despite the similar kinetics of translocation across the chloroplast envelope membranes for all of the C-terminally truncated precursors, the overall efficiency of their import into the stroma was reduced, particularly for the most truncated forms (OE23–1D and OE23–1E).

It is noteworthy that several of the truncated OE23 intermediates persisted in the stroma longer than the full-length OE23 intermediate. In contrast to the almost complete chase of the wild-type intermediate form into the thylakoid lumen, intermediates of both OE23–1B and OE23-1C could be observed at the 20-min point, and the OE23–1A intermediate was present at the 10-min point. These results suggest that translocation across the thy-

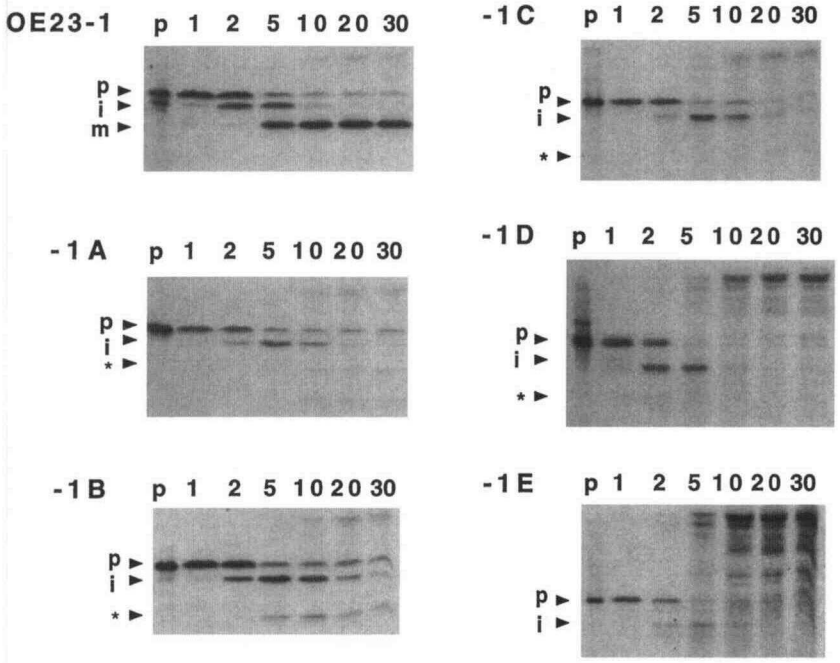

Figure 3. Import kinetics of OE23–1 and truncated OE23–1 precursors into chloroplasts. Import reactions were initiated in the light as described for Figure 2. Aliquots were removed at the indicated times (minutes) and centrifuged through silicon oil into 1.5 M perchloric acid. Polypeptides were visualized by SDS-PAGE and fluorography. p, Precursor OE23; i, intermediate OE23; m, mature OE23; asterisk, low-molecular-weight OE23.

lakoid may be inhibited as a result of the loss of as few as 10 amino acids from the carboxyl end of the precursor. On the other hand, the intermediates detected for OE23 precursors with the largest truncations, OE23–1D and OE23–1E, disappeared at rates similar to that seen with the full-length intermediate. These differences in the rates of turnover of the various intermediates suggest the possibility of stringent conformational requirements for efficient translocation of OE23 across the thylakoid membrane.

In the time-course experiments shown in Figure 3, lower molecular weight forms of the truncated proteins were observed between 2 and 10 min of import. OE23 protein fragments of 14 to 16 kD appeared in small amounts in the OE23–1A and OE23–1B samples (indicated by the asterisk in Fig. 3). The OE23–1C and OE23–1D import experiments resulted in the appearance of extremely faint, low-molecular-weight bands that may represent mature, processed OE23 in the lumen. None of the low-molecular-weight forms accumulated to amounts high enough to account for the level of stromal intermediate observed during the import time course. These low-molecular-weight proteins were smaller than those expected by the truncations alone and appear to have arisen by aberrant processing in the lumen. It is doubtful that these proteins passed through a "mature size" stage, since the time-course reactions were terminated by centrifuging the samples through oil into perchloric acid. This technique is best suited for detection of transient polypeptide species, because the acid precipitation step inhibits further translocation or protease activity that might affect the distribution between precursor, intermediate, or mature forms of the protein. The small amount of low-molecular-weight protein that can be observed after 5 to 10 min of import of the OE23–1A, OE23–1B, OE23–1C, and OE23–1D precursors did not accumulate significantly and could hardly be detected by the 30-min point. This suggests that degradation of these polypeptides occurred rapidly on the time scale of the import experiment. These results are in sharp contrast to observations showing the mature, full-length OE23 protein to be extremely stable in the soluble lumen pool (Ettinger and Theg, 1991) with a half-life of at least 8 h in isolated chloroplasts (A. Hashimoto, unpublished data).

Stability of the Truncated Intermediates in the Stroma

Since the truncated mature proteins did not appear to accumulate during the import time course, it was unclear whether the disappearance of their stromal intermediate forms over time was due to their translocation into the thylakoid or to their degradation in the stromal compartment. To address this question, import time-course experiments were carried out for the OE23–1A and OE23–1B precursors without illumination in the presence of exogenous ATP, and with the addition of the ionophores nigericin and valinomycin to eliminate the pH gradient across the thylakoid membrane. Under these conditions, import of precursors into chloroplasts is possible, but thylakoid translocation is completely blocked (Cline et al., 1992). As shown in Figure 4, the OE23–1A and OE23–1B truncated intermediates were observed throughout the 30-min time course, indicating that these intermediates are stable in the stromal compartment and are not degraded by stromal proteases. The apparent degradation of the protein is therefore most likely to occur during translocation across the thylakoid membrane or within the thylakoid lumen following translocation.

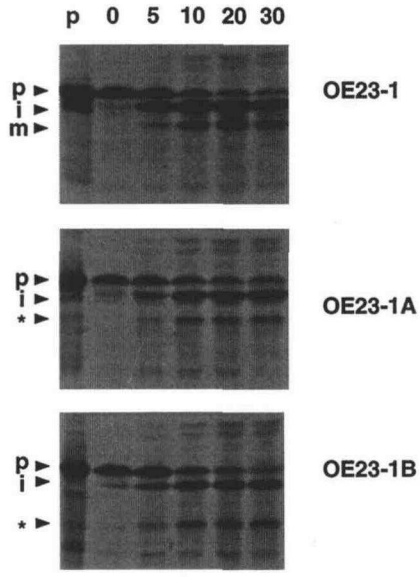

Figure 4. Stromal intermediate forms of full-length and truncated OE23s are stable during 30 min of incubation in the dark. Import reactions and sample treatment are as in Figure 3, except that the *ionophores nigericin and valinomycin were added to the import* media, and the import reactions were kept in the dark at room temperature for the times indicated (0, 5, 10, 20, and 30 min) following 5 min of import in the light. p, Precursor OE23; i, intermediate OE23; m, mature OE23; asterisk, low-molecular-weight OE23.

Subchloroplast Fractionation of the Truncated Intermediates

Unlike the normal, full-length intermediate, which is invariably found in the soluble stromal fraction, a significant proportion of the truncated OE23–1A and OE23–1B intermediates appeared to be associated with the thylakoid membrane following an osmotic lysis of the chloroplasts (Fig. 5). This was true even when the import reactions were performed in the presence of uncouplers that inhibit thylakoid translocation of the full-length intermediate (Fig. 5B). As shown in Figure 6, the membrane-bound intermediates, which could be completely degraded by external proteases, were not released by washing with buffer, and were only partially solubilized in 500 mM NaCl or 0.1% Triton X-100. They were completely released from the membrane fraction by treatment with urea. It appears from these data that the normal interaction of the OE23 intermediate with the thylakoid membrane transport machinery is altered by the loss of the carboxyl terminus, resulting in a form that strongly interacts with the outside of the membrane.

Translocation Competence of Membrane-Bound Intermediates

To assess if these membrane-bound forms of intermediate OE23 were simply stuck on the membrane or if they represented translocation-competent, "stalled" intermediates on the import pathway, a pulse-chase experiment was carried out using the OE23–1A-truncated precursor. Chloroplast import reactions were carried out with exogenous ATP in the presence of the ionophore CCCP, which allows the accumulation of the stromal intermediate by blocking translocation across the thylakoid membrane. As can be seen in the control reactions in Figure 7A, some protein translocation across the thylakoid membrane occurred during the 15-min dark import (seen as mature bands in lanes 3 and 5), but a significant portion accumulated as the stromal intermediate. The full-length OE23–1 intermediate was found in the soluble stromal fraction following chloroplast subfractionation (Fig. 7, lane 2). Both an intact chloroplast aliquot (Fig. 7, lanes 5 and 6) and a thylakoid membrane aliquot (Fig. 7, lanes 3 and 4) derived from re-isolated chloroplasts from the initial dark reaction were subjected to normal import conditions for 15 min under illumination in the presence of BSA. BSA has been shown to adsorb CCCP with high avidity, effectively removing the uncoupler and allowing the intermediate to chase across the thylakoid membrane when the pH gradient is reestablished by illumination (W.F. Ettinger and S.M. Theg, unpublished results; Creighton et al., 1995). For the full-length OE23 (Fig. 7A), this chase was observed in the chloroplast samples as the disappearance of the ac-

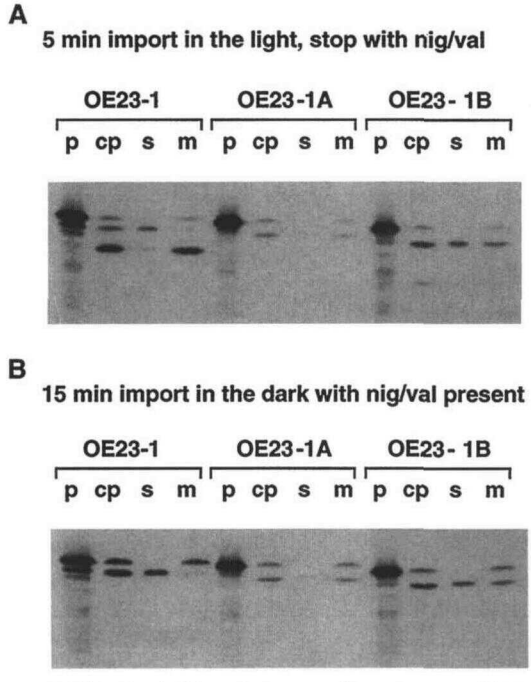

Figure 5. Unlike the full-length intermediate, truncated intermediates fractionate with thylakoid membranes. Reactions were run either in the light for 5 min (A) or in the dark for 15 min in the presence of nigericin (nig) and valinomycin (val) (B). Reactions were terminated by placing the samples on ice in the dark; samples from the experiment in A also received 2 μM nigericin and valinomycin at this time. Chloroplasts were re-isolated by pelleting through a 40% Percoll cushion; the pelleted chloroplasts were analyzed directly (chloroplast, cp) or after osmotic lysis and generation of stromal (s) and membrane (m) fractions.

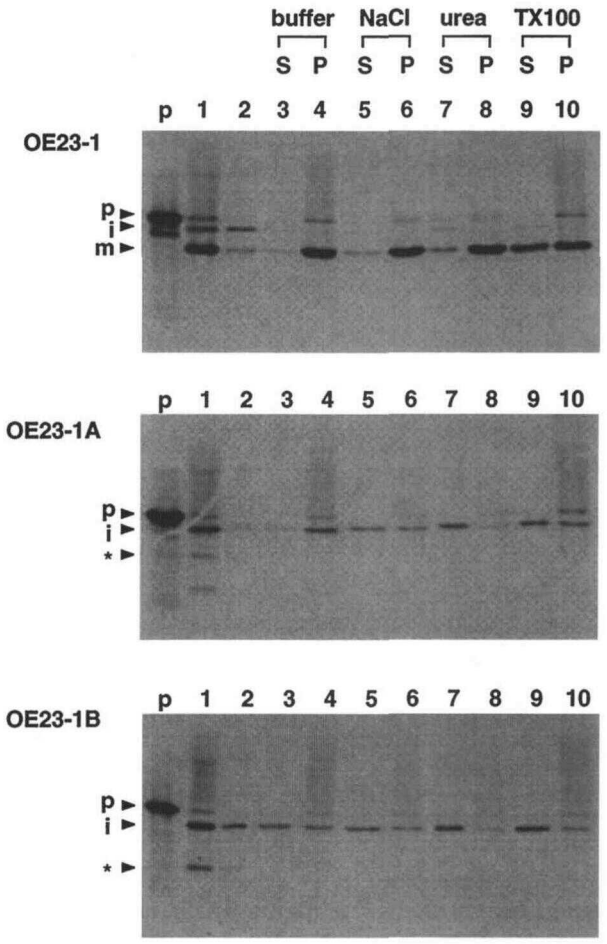

Figure 6. Characterization of the membrane-binding properties of the OE23–1A and OE23–1B intermediates following 5 min of import into chloroplasts. Import reactions were terminated after 5 min in the light by the addition of nigericin and valinomycin and then placed immediately on ice in the dark. Chloroplasts (lane 1) were subsequently separated into soluble (lane 2) and membrane (lanes 3–10) fractions by osmotic lysis. The membrane fraction was separated into four aliquots, which were incubated for 15 min on ice in buffer (20 mM Suc, 20 mM Mes, pH 6.5, and 5 mM $MgCl_2$) or in buffer containing NaCl (0.5 M NaCl), urea (1.3 M urea and 0.1 M NaCl), or TX100 (0.1% Triton X-100). Soluble and membrane fractions were recovered as supernatant (S) and pellet (P) following centrifugation at 10,000*g* for 5 min. p, Precursor OE23; i, intermediate OE23; m, mature OE23; asterisk, low-molecular-weight OE23.

cumulated stromal intermediate. The intermediate derived from the truncated protein (Fig. 7B) also was observed to decrease during the chase in both the chloroplast sample and in the thylakoid membranes. Since the former sample contained both bound and soluble intermediate, whereas the latter contained only the membrane-bound intermediate, these results suggest that during the chase in intact chloroplasts the soluble truncated intermediate was translocated in addition to the membrane-bound species. These results suggest that at least some of the membrane-bound population of the truncation intermediate is tightly associated with the membrane in a productive manner following de-energization of the thylakoids. This tight binding was not observed for the full-length OE23 intermediate and appears to be a consequence of the removal of the carboxyl portion of the protein.

Protease Susceptibility of the Truncated Precursors

The unusual binding of the OE23–1A intermediate to the thylakoid in the stromal compartment and the rapid degradation of the truncated polypeptides in the lumen suggest that some alteration in the overall conformation of the protein occurred as a result of these rather minimal C-terminal deletions. As a means to detect changes in protein conformation, we subjected the full-length and the OE23–1A (Fig. 8) and OE23–1B (data not shown) truncated precursors to digestion with endogenous proteases. In such experiments, any change in protease susceptibility between these species is indicative of a conformational difference. The result of trypsin digestion shown in Figure 8A revealed the loss of a characteristic protease-resistant fragment of the full-length OE23 protein in the truncated OE23–1A polypeptide (Creighton et al., 1995). Altered sensitivity to the proteases proteinase K and thermolysin were also observed (Fig. 8, B and C). Although protease-resistant fragments were observed in the truncated precursors,

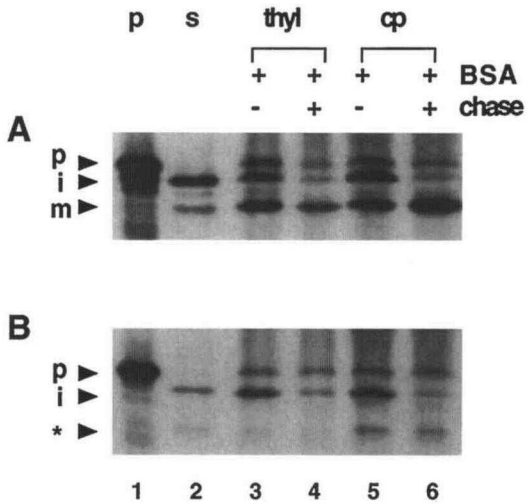

Figure 7. Translocation of intermediate OE23 following removal of CCCP. Import reactions into isolated chloroplasts were initiated in the dark in the presence of 10 μM CCCP. A and B were performed with prOE23–1 and prOE23–1A, respectively. In lanes 2 to 4, reisolation and subfractionation of chloroplasts were performed as in Figure 2, except that all buffers used throughout the procedure contained 2 μM CCCP. s, Stromal fraction; thyl, thylakoid fraction. The lanes labeled "+ BSA/+ chase" were illuminated for 15 min following the addition of 2.5 mg BSA/mL; the samples labeled "+BSA/− chase" were kept on ice in the dark for 15 min. The last two lanes (chloroplast, cp) show the results of the chase experiment performed without prior fractionation of the plastids. p, Precursor OE23; i, intermediate OE23; m, mature OE23; asterisk, low-molecular-weight OE23.

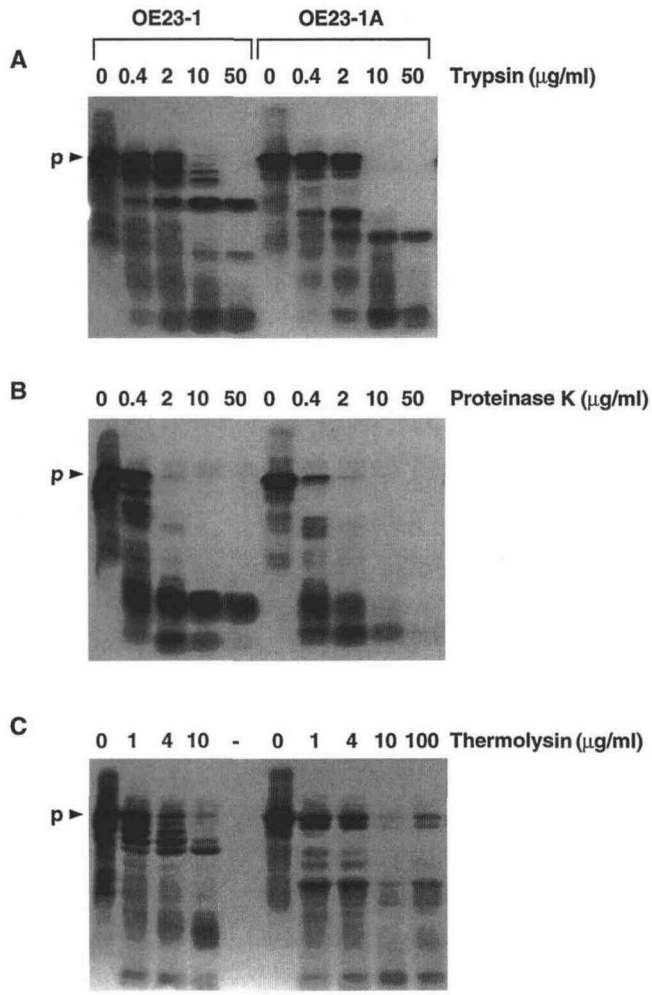

Figure 8. Truncated OE23–1A precursor is more susceptible than full-length OE23–1 precursor to degradation by exogenous proteases. The in vitro translated precursors were incubated for 20 min on ice with the indicated concentrations of the indicated proteases. Reactions were terminated by the addition of 0.2 mg/mL trypsin inhibitor (A), by the addition of 2 mM PMSF (B), or by the addition of 15 mM EDTA (C). p, Precursor OE23.

they were different fragments than those generated with the full-length precursor. Therefore, these data demonstrate that the various species of OE23 precursors possess different conformations. These conformational changes caused by the loss of C-terminal regions of the OE23 precursor are likely to be the cause of the altered behavior of the truncated OE23 subunits toward thylakoid transport, stability in the lumen, and assembly into the OEC.

DISCUSSION

C-terminal end of the OE23 precursor protein appears to play a critical role in the proper targeting of this protein to the thylakoid lumen. In addition, it would appear that the deletion of as few as 10 or as many as one-half of the amino acids in the mature protein has a similar effect on translocation and assembly of OE23. The lack of assembly of the truncated subunits into PSII seems to stem from two factors: (a) inefficient translocation across the thylakoid membrane and (b) the instability of the translocated and processed form once within the lumen.

The translocation of truncated intermediates occurs over a time range that is similar to the full-length intermediate in that the first appearance of processed, low-molecular-weight forms of OE23 in the lumen occurs within 2 to 5 min of the onset of energized chloroplast import (Fig. 3). The intermediate forms, however, are observed over the course of about 20 min rather than disappearing after a few minutes, which is the case for the full-length OE23. The exceptions to this pattern are the most severely truncated forms of the OE23 intermediate, which disappear altogether within 10 min. Since the OE23–1A and OE23–1B intermediates were shown to be stable in the stroma during dark incubation of chloroplasts in the presence of ionophores (Figs. 4, 5B, and 7), we assume that the eventual disappearance of these forms from the stroma in the light is the result of translocation across the thylakoid membrane and immediate proteolytic degradation.

Given the unusual behavior of the truncated proteins, we wondered if the truncations could be shown to cause significant alterations in the conformation of the precursor, and by extension, the intermediate that is the substrate for thylakoid translocation. We found that removal of as few as the last 10 amino acids, a truncation we expected would have little impact on the folding of the protein, did indeed cause a conformational change that could be detected by protease experiments (Fig. 8). The nature of the conformational change could not be determined by these techniques; however, we do not believe that our results can be explained simply by the hypothesis that the loss of C-terminal residues rendered the thylakoid transit signal inaccessible to the transport machinery. It is clear from our kinetics experiments that some of the truncated forms of at least OE23–1A and OE23–1B did reach the lumen. Indeed, the first appearance of the lumen forms of these proteins coincided with the appearance of the mature, full-length OE23–1, showing that the kinetics of the translocation event were not altered per se. What did appear to be changed was the amount of truncated protein that interacted productively with the translocation machinery at any given moment. Ultimately, all of the intermediate-sized truncated proteins disappeared over 20 to 30 min. Since the stromal intermediate species were determined to be stable, we interpret this result to indicate that all of the intermediate was transported to the lumen and then degraded.

The most likely explanation of our data is that the aberrant behavior of the truncated precursors is the result of their putative altered conformation. This is an unexpected interpretation in light of the paradigm that proteins must be unfolded to a "looser" (Hannavy et al., 1993) or even a fully extended (Rassow et al., 1990) conformation during membrane transport (Schatz and Dobberstein, 1996). In view of this, we might have expected the truncated proteins to be more readily transported than the full-length iOE23, since the increased protease sensitivity of these precursors indicates that they are in a less constrained or

more loosely folded conformation. In fact, we observed just the opposite, which suggests that the altered conformation is not tolerated by the thylakoid translocation apparatus as well as the native conformation. This suggests that structural motifs present in the passenger protein are important determinants of productive membrane interactions (cf. Ko and Ko, 1992), and that perhaps the protein is transported in its folded state. We note that Creighton et al. (1995) were unable to demonstrate any unfolding of the iOE23 in the stroma; these authors concluded that it is either unfolded during transport or not unfolded at all. Other precedents exist for the transport of proteins across biological membranes in their completely folded conformations (Glover et al., 1994; McNew and Goodman, 1994; Walton et al., 1995), raising the question of whether the requirement for polypeptide unfolding for membrane traversal applies only to specific membranes and/or proteins.

The aberrant processing of the truncated proteins in the thylakoid lumen might also be taken as evidence for transport of iOE23 species in a folded conformation. The cleavage site for the thylakoid processing protease is well defined (A-X-A; Halpin et al., 1989) and was present in all of our truncated constructs. Furthermore, this protease must act almost immediately upon entry of the proteins into the lumen, because we have never detected, nor have others reported, uncleaved proteins in this space. Therefore, the aberrant processing of the truncated proteins might reflect inaccessibility of the cleavage site as these proteins enter the lumen. This would not be expected if proteins enter the lumen N termini first and in an extended conformation. The possibility that proper cleavage is followed quickly by further degradation cannot be completely ruled out by our experiments. However, we found no evidence for this interpretation in our time-course experiments, in which "snapshots" of the progression of the proteins through the chloroplasts were obtained by pelleting the samples through oil into denaturing acid. The entire termination processes took no more than 15 s.

Subchloroplast fractionation showed that a portion of the truncated stromal intermediates was bound to the thylakoid membrane following treatments that caused their accumulation (addition of ionophores). In contrast, the stromal intermediate form of the full-length precursor is invariably observed in the soluble stromal fraction following chloroplast lysis. Because as much as 50% of the OE23–1B stromal intermediate was bound to the thylakoid membrane, we questioned whether this bound species was interacting productively with the translocation apparatus. In experiments designed to allow the accumulation of the stromal intermediate to be separated in time from translocation across the thylakoid membrane, we found that much of the membrane-bound form of the stromal intermediate could be chased into the thylakoids. Our experiments do not allow us to determine whether these membrane-bound truncated intermediates are associated with the translocation machinery or simply stuck to the membrane surface nonspecifically in a location from which they move into transport sites. The fact that these "stuck" proteins washed off the membranes with urea, but not with NaCl or detergent (Fig. 5), suggests that the membrane association is likely to involve hydrophobic interactions. It seems reasonable to suppose that binding of the intermediates to the thylakoid membrane is another manifestation of the altered structures of the truncated forms of the iOE23, and again suggests that the protein is not translocated in an extended conformation.

A difficulty is encountered with the interpretation that the pH gradient-dependent transporter responsible for iOE23 translocation into the thylakoid lumen requires the native conformation of the substrate polypeptide when one considers that other passenger proteins can be made to follow this pathway by attachment of the appropriate transit peptide (Henry et al., 1994; Robinson et al., 1994). This difficulty can be met by relaxing the requirement for particular structures to allow only a bias toward specific structural components. In this manner, it can be postulated that these structural components are present in the full-length OE23 and some other polypeptides, but not in the truncated constructs that we have examined here.

In our view, the most remarkable observation made during the course of these studies was the extreme instability of the truncated proteins in the thylakoid lumen. We often found the truncated proteins to be undetectable after import reactions lasting 20 to 30 min. This is in contrast to the wild-type OE23, which survives, even in the unassembled soluble pool present in the lumen (Ettinger and Theg, 1991), with a half-life of greater than 8 h (A. Hashimoto, unpublished data). This drastic reduction in lifetime is even more remarkable when one considers the minimal nature of the truncation; OE23–1A, for instance, is missing only 10 amino acids from its carboxyl terminus. Chloroplasts are known to contain proteases that quickly degrade subunits that are not immediately assembled (Bennett, 1981; Schmidt and Mishkind, 1983; Merchant and Selman, 1984), and some of these function in the thylakoid lumen (Merchant and Bogorad, 1986; Kuwabara and Suzuki, 1994; Li and Merchant, 1995). Although this appears to be a general mechanism for the regulation of subunit stoichiometry in protein complexes (Luzikov, 1986), the subunits of the OEC do not follow this pattern of assembly or degradation (Ettinger and Theg, 1991). It has always remained a possibility that the unassembled OEC subunits escape proteolytic degradation because they are simply not recognized by the rather active proteases present in the lumen. Our present studies would seem to indicate that these subunits are not intrinsically unrecognizable, but rather protected, presumably by their correct conformations. Without the appropriate final structure, the OE23 is treated like any other unassembled or aberrantly folded polypeptide and subjected to immediate degradation.

The rapid turnover of the truncated OE23 subunits in the thylakoid lumen prevented us from determining whether they are competent for assembly into the OEC. The fact that we never observed subunits that had entered the lumen in association with membrane fractions suggests that they are not. However, we cannot rule out the possibility that once assembled, the OE23–1A and OE23–1B subunits were susceptible to degradation from a position within the OEC.

Nor could we assign assembly incompetence to the missing carboxyl terminus, since the processing protease that removes the transit peptide in the lumen apparently cleaved the protein at the wrong site, resulting in subunits with alterations at both ends. It will be possible to resolve this question, however, with the more conventional approach of reconstitution of OEC subunits to stripped PSII particles. Such experiments are currently under way in our laboratory.

ACKNOWLEDGMENTS

The authors would like to thank Ellen Leheny for critical reading of the manuscript and Akiko Hashimoto for permission to cite unpublished results.

Received November 20, 1995; accepted May 9, 1996.

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