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# Maturation of thylakoid lumen proteins proceeds post-translationally through an intermediate *in vivo*

(*Chlamydomonas reinhardtii*/protein import/post-translational processing/cytochrome *c6*)

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ABSTRACT **Many thylakoid lumen proteins are synthesized outside the chloroplast as larger molecular weight precursors and then processed to their mature size during transport to the lumenal space. We have examined the post-translational processing of thylakoid lumen proteins *in vivo* by pulse-radiolabeling experiments with *Chlamydomonas reinhardtii*. Antibodies against the lumenal protein cytochrome *c6* specifically immunoprecipitated three polypeptides from extracts of briefly pulse-radiolabeled cells. The molecular weights and kinetics of synthesis and turnover indicate that these three polypeptides are (*i*) the full-length cytochrome *c6* precursor, (*ii*) a partially processed precursor (intermediate), and (*iii*) the completely processed mature protein. Identification of analogous forms of two other lumenal proteins, plastocyanin and the oxygen evolving enhancer 1 protein, indicates that the maturation of thylakoid lumen proteins occurs post-translationally *in vivo* and that the partially processed intermediate is a general feature of the pathway. The intermediate form of cytochrome *c6* accumulated to a greater extent in cells incubated at 10°C, relative to cells incubated at 22°C, concomitantly with a decrease in the accumulation of the mature protein. The intermediate accumulating at 10°C is quantitatively converted to the mature protein upon incubation at higher temperature, thus demonstrating a precursor–product relationship between the intermediate and mature forms of cytochrome *c6*. Our results prove the model [Smeekens, S., Bauerle, C., Hageman, J., Keegstra, K. & Weisbeek, P. (1986) *Cell* 46, 365–375] that precursors of lumenal proteins are post-translationally converted to their mature forms in two steps through a distinct intermediate.**

Most proteins located in the thylakoid lumen of the chloroplast are synthesized outside the organelle with an amino-terminal presequence. The presequences of these lumenal proteins consist of two functional domains that assist in targeting the protein across the chloroplast envelope (envelope transfer domain) and thylakoid membranes (thylakoid transfer domain) (1–3). Based on *in vitro* studies of the biosynthesis of the thylakoid lumen protein plastocyanin, it was originally proposed that the presequence is cleaved in two steps during the import of precursors into intact chloroplasts (4). According to this model, the precursor is translocated across the chloroplast envelope, and then the envelope transfer domain of the presequence is removed by a stromal peptidase to generate an intermediate. Following translocation of the intermediate across the thylakoid membrane, a second processing step removes the thylakoid transfer domain, thus generating the mature-sized protein in the lumen.

The central feature of the two-step import model—namely, that the intermediate species is the substrate for the second translocation and processing steps—remains to be proven. The model was proposed on the basis of the appearance of an intermediate form of plastocyanin in the stroma during the import of preplastocyanin into intact chloroplasts (4). Since that time, several studies have attempted to establish a precursor–product relationship between the putative intermediate and mature forms of several thylakoid lumen proteins to substantiate this model (5–9). However, the results of these studies are conflicting. For example, in a kinetic study of the transport of thylakoid lumen proteins *in vitro* (6), it was concluded that "the accumulation patterns observed for these soluble intermediate sized forms depended on incubation conditions and could not be consistently explained by a simple model where the intermediate sized form is the substrate for the second step. Thus it has not been possible to clearly identify the substrate for thylakoid translocation *in organello*." In another work (8), "the results indicate that the thylakoidal protein transport system can import both the precursor and intermediate forms of the 23-kDa protein" and our interpretation of the data presented in that work indicates that the precursor is translocated across the thylakoid membrane more efficiently than is the putative intermediate. The *in vitro* work is further complicated by the presence of multiple "intermediate species" generated during the reaction (5–8). Finally, although full-length precursors of all lumenal proteins are good substrates for translocation across purified thylakoid membranes (7–10), the intermediate forms of at least two of these proteins are not (5, 7). These findings raised the possibility that the precursors of lumenal proteins are directly processed to their mature forms without involvement of a stromal processing step, and it was suggested that the intermediate forms of lumenal proteins observed *in vitro* are "dead-end" products rather than *bona fide* kinetically competent intermediates in the import pathway (6).

To resolve these ambiguities we undertook to examine the post-translational maturation of lumenal proteins *in vivo*. Cytochrome *c6* (cyt *c6*) of *Chlamydomonas reinhardtii*, like other thylakoid lumen proteins, is encoded by a single nuclear gene and is synthesized outside the chloroplast with a "two-domain" presequence at its amino terminus (11). The maturation of pre-apocyt *c6* to its mature form involves several post-translational steps, including translocation of the precursor across the chloroplast envelope and thylakoid membranes, processing of the presequence, and covalent ligation of heme to the apoprotein (12). Once synthesized, holocyt *c6* is a soluble protein functioning in the thylakoid lumen as a catalyst of photosynthetic electron transfer between the cytochrome *b6f* complex and photosystem I. In *C. reinhardtii*, this reaction can also be catalyzed by the copper protein plastocyanin. The utilization of cyt *c6* vs. plastocyanin depends upon the availability of copper in the medium (13). If the concentration of copper in the medium is sufficient

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Abbreviations: cyt *c6*, cytochrome *c6*; OEE1, oxygen-evolving enhancer 1 protein.

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to maintain continued assembly of holoplastocyanin, transcription of the cyt *c6* gene is completely repressed (14). In copper-depleted cells, where apoplastocyanin is continually synthesized but rapidly degraded (15), the cyt *c6* gene is transcribed and the cells use the hemoprotein as a "standby" electron transfer catalyst. Since the expression of cyt *c6* is conveniently manipulated by provision or depletion of medium copper ions, genuine *in vivo* intermediates are distinguished by comparative studies in copper-supplemented vs. copper-deficient cells.

We report here on the post-translational processing of cyt *c6* and other lumenal proteins *in vivo*. Our results show (*i*) that the precursor of cyt *c6* is a definable species in the pathway *in vivo* and (*ii*) that it is processed to the mature protein in two steps through a short-lived intermediate. We propose that precursor and intermediate molecules are general features of the pathway for the maturation of thylakoid lumen proteins *in vivo*.

## MATERIALS AND METHODS

**Growth and Labeling of Cells.** *C. reinhardtii* wild-type strain (CC-124) was obtained from the *Chlamydomonas* Genetics Center, Duke University, Durham, NC. Cells were cultured at 25°C under a light intensity of 125 microeinsteins per $m^2/s$ in Tris/acetate/phosphate (TAP) medium in which the copper concentration was reduced to <3 nM and the sulfate concentration was reduced to 95 $\mu$M by using $MgCl_2$ in place of $MgSO_4$ (12). A concentrated cell suspension ($10^8$ cells per ml) was prepared for labeling as described (12, 16). Labeling was initiated by the addition of $Na_2{}^{35}SO_4$ (carrier-free, 1050–1600 Ci/mmol, 1 Ci = 37 GBq; NEN) to a final concentration of 1.5 mCi/ml. The average rates of labeling, determined by measuring the incorporation of $^{35}S$ into trichloroacetic acid-insoluble material (12), were approximately 0.41 and 0.14 cpm per cell per min at 22°C and 10°C, respectively. The "chase" period was initiated by the addition of $Na_2SO_4$ and cycloheximide to final concentrations of 25 mM and 50 $\mu$g/ml, respectively.

**Preparation of Cell Extracts and Immunoprecipitation.** Proteins were extracted from labeled cells by removing 0.125 ml of the labeled cell suspension into 1.0 ml of 100% acetone at 0°C. Acetone precipitates were collected, redissolved, and boiled in 0.1 ml of 2% SDS/60 mM Tris·HCl, pH 8.6/60 mM dithiothreitol/5 mM $\varepsilon$-aminocaproic acid/5 mM benzamidine/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride. Solubilized extracts were diluted 5-fold with immunoprecipitation buffer (150 mM NaCl/40 mM Tris·HCl, pH 7.5/2% Nonidet P-40/2 mM EDTA) and immunoprecipitated as described (12) with polyclonal antibodies raised against holocytochrome *c6*, oxygen-evolving enhancer 1 protein (OEE1), and plastocyanin of *C. reinhardtii*. Antibody titering experiments showed that 20 $\mu$l of antiserum was sufficient to quantitatively immunoprecipitate (>90%) the antigen of interest from extracts derived from $1.25 \times 10^7$ wild-type cells. To reduce the amount of nonspecific binding during immunoprecipitation of cyt *c6* or OEE1, dissociated immunoprecipitates were diluted 5-fold with immunoprecipitation buffer and subjected to a second round of immunoprecipitation with the same antibody.

**Gel Electrophoresis.** For gel electrophoresis, immunoprecipitates were dissociated in 20 $\mu$l of a solution containing 3% SDS, 62.5 mM Tris·HCl (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 0.1% bromphenol blue and heated in a boiling water bath for 5 min. Undissolved material was removed by centrifugation (12,000 × *g*, 2 min) and the resulting supernatant was analyzed by polyacrylamide gel electrophoresis (10–17% polyacrylamide gradients for cyt *c6* and plastocyanin or 10–15% gradients for OEE1) and fluorography. Laser scanning densitometry (Bio-Rad, model 620) was used for the quantification of relevant signals on the fluorographs.

**Protein Standards.** $^{35}S$-labeled holocytochrome *c6* was prepared as described (12). Pre-apocyt *c6* and pre-apoplastocyanin were prepared by *in vitro* transcription and translation (rabbit reticulocyte lysate) of the respective cloned cDNAs (11, 17). Pre-OEE1 was immunoprecipitated from the products of a cell-free wheat germ translation reaction programed with total RNA from a wild-type strain as described (12). The apparent molecular weights of the different forms of cyt *c6*, plastocyanin, and OEE1 were estimated by plotting their electrophoretic mobility relative to the mobility of radiolabeled protein standards.

## RESULTS

The widely accepted notion that precursor proteins are translocated into chloroplasts by a post-translational event (18) implies the presence of a pool of precursor substrates in the cytoplasm. In the case of lumenal protein biogenesis, the two-step model further proposes that a partially processed precursor (intermediate) is generated in the stroma during import (Fig. 1*A* and ref. 4). For the further elaboration and confirmation of the pathway, identification of these species *in vivo* is necessary. Nevertheless, unprocessed precursors and partially processed intermediates remain elusive *in vivo*. Since rapid turnover of these molecules would prevent their facile detection, an experimental system where proteins could be very briefly pulse-radiolabeled and extracted under conditions that prevent their degradation or turnover was necessary. Therefore, a copper-deficient (cyt *c6*-expressing) culture of *C. reinhardtii* was pulse-labeled with $Na_2{}^{35}SO_4$ for

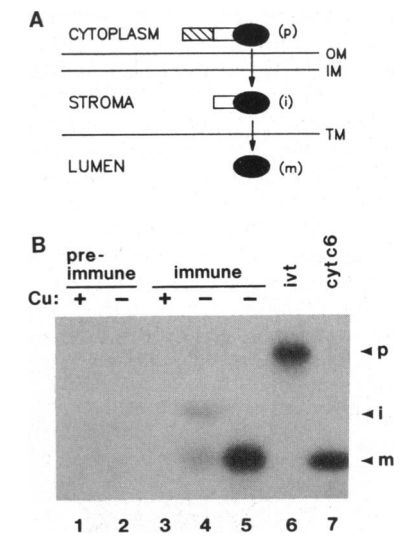


FIG. 1. (*A*) Proposed pathway for the biosynthesis of thylakoid lumen proteins (4). See text for details. The two domains of the presequence at the amino terminus of the protein are represented as a hatched bar (envelope transfer domain) and an open bar (thylakoid transfer domain). The filled oval represents the mature portion of the protein. OM, outer membrane; IM, inner membrane; TM, thylakoid membrane; p, precursor; i, intermediate; m, mature. (*B*) Identification of three electrophoretically distinguishable forms of cyt *c6* *in vivo*. Cultures of *C. reinhardtii* grown in either the presence (+) or absence (−) of medium copper were labeled with $Na_2{}^{35}SO_4$ for 2.5 min at 22°C. Cells were then immediately extracted with acetone (lanes 1–4) or were incubated (chased) for 5 min in the presence of unlabeled $Na_2SO_4$ and cycloheximide prior to acetone extraction (lane 5). Acetone extracts were solubilized and immunoprecipitated with anti-cyt *c6* antibodies or preimmune serum, as indicated. Immunoprecipitates were analyzed by denaturing polyacrylamide gel electrophoresis and fluorography. Lane 6, pre-cyt *c6* synthesized *in vitro* (ivt); lane 7, purified holocyt *c6*. p, i, and m, as in *A*.

2.5 min and then extracted with acetone to stop further protein labeling and degradation. To identify putative precursors and intermediates in the maturation of thylakoid lumen protein cyt *c6*, the acetone precipitate was solubilized and then immunoprecipitated with polyclonal antibodies raised against holocyt *c6*. Three forms of cyt *c6* were observed following separation of the immunoprecipitate by gel electrophoresis (Fig. 1*B*, lane 4). The specificity of the immunoprecipitation reaction was established by the fact that none of these three polypeptides was immunoprecipitated with preimmune serum (lanes 1 and 2) nor were they immunoprecipitated with anti-cyt *c6* serum from extracts of cells grown in Cu-supplemented medium—i.e., cells that do not express cyt *c6* (lane 3). The lowest molecular weight form of cyt *c6* that is immunoprecipitated from pulse-labeled cells is the completely processed (mature) protein as determined by amino-terminal sequencing (not shown). [The heme-containing protein (holocyt *c6*) and the heme-deficient protein (apocyt *c6*) migrate at the same position on this gel system (12)]. The other two forms of cyt *c6* immunoprecipitated by anti-cyt *c6* have apparent $M_r$s of 15,000 and 8000. The larger of these polypeptides comigrates with pre-apocyt *c6* synthesized *in vitro* (lane 6) and is therefore a good candidate for the authentic full-length precursor to cyt *c6*. Thus, the polypeptide of $M_r$ 8000 is an "intermediate" between the precursor and mature forms of cyt *c6*. The full-length precursor and intermediate forms of cyt *c6* were not observed in cells pulse-labeled for 2.5 min and then incubated (chased) with unlabeled $Na_2SO_4$ and cycloheximide (Fig. 1*B*, lane 5), indicating that these two polypeptides, but not mature cyt *c6*, are rapidly turned over *in vivo*.

To establish a time course for the onset of accumulation of the three forms of cyt *c6*, cells were very briefly pulse-labeled with $Na_2{}^{35}SO_4$ for various times (Fig. 2*A*). Under these experimental conditions, an initial lag period of 0.5–1 min was noted before incorporation of the isotope into protein was detected (not shown). At the earliest time points (1 min), the precursor and the intermediate forms of cyt *c6* were labeled prior to mature protein, suggesting that their synthesis in the cyt *c6* maturation pathway precedes that of the mature protein. Although it was not possible in wild-type cells to label the precursor in the complete absence of labeling of the intermediate, in some experiments [or in experiments with the B6 mutant (12)] we observed that the precursor was labeled much more heavily than the intermediate in cells subjected to a very brief pulse label (data not shown),

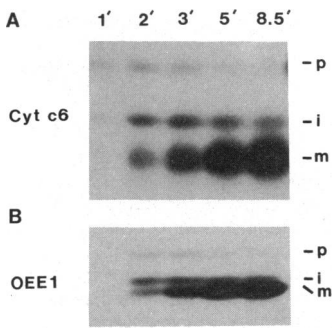


FIG. 2. Kinetics of labeling of cyt *c6* and OEE1 *in vivo*. A culture of *C. reinhardtii* was incubated with $Na_2{}^{35}SO_4$ at 22°C and thereafter, at the times indicated (min), samples of the labeling reaction were quenched into acetone. Solubilized acetone extracts were immunoprecipitated with antibodies against either cyt *c6* (*A*) or OEE1 (*B*). Immunoprecipitates were analyzed by SDS/polyacrylamide gel electrophoresis and fluorography. The apparent $M_r$s of the precursor (p), intermediate (i), and mature (m) forms of cyt *c6* are 15,000, 8000, and 5000, respectively. The apparent $M_r$s of the analogous forms of OEE1 are 32,000 (p), 30,000 (i), and 29,000 (m).

suggesting that the precursor is indeed synthesized prior to the intermediate form. Further evidence for this is that the steady-state level of labeling of the precursor appeared to be established before that of the intermediate (compare the 1- and 2-min time points, Fig. 2*A*). Thereafter, the mature protein was labeled heavily with increasing time, indicative of the stable accumulation of holocyt *c6*. These results are consistent with the model that the three forms of cyt *c6* are synthesized in the order precursor → intermediate → mature.

The identification of precursor and intermediate forms of cyt *c6* prompted us to examine whether analogous species of other nuclear-encoded lumenal proteins are also synthesized *in vivo*. Two of the best-characterized lumenal proteins in *C. reinhardtii* and in vascular plants are the OEE1 and plastocyanin. In *C. reinhardtii*, OEE1 and plastocyanin are synthesized with a typical two-domain presequence that is cleaved during the transport of the precursor to the thylakoid lumen (17, 19). Polyclonal antibodies raised against OEE1 immunoprecipitated three polypeptides with apparent $M_r$ of 29,000, 30,000, and 32,000 from extracts of pulse-labeled cells (Fig. 2*B*). The fastest migrating polypeptide ($M_r$ 29,000) was identified as the mature form of OEE1 as evidenced by its continuing accumulation following *de novo* synthesis (Fig. 2*B*) and its comigration with the mature protein (not shown), whereas the slowest migrating form ($M_r$ 32,000) was identified as the OEE1 precursor protein based on its comigration with pre-OEE1 synthesized *in vitro* (not shown). The intermediate species ($M_r$ 30,000) is thus identified as a partially processed precursor to mature OEE1. It was not possible to discern an order of labeling in this case because the initiation of synthesis of the three forms of OEE1 was kinetically indistinguishable. At the earliest time point where labeling of OEE1 was detectable (2 min), all three forms of the protein were labeled. Thereafter, the mature protein accumulated rapidly while the levels of precursor and intermediate remained relatively constant (i.e., attained a steady state), as observed for cyt *c6* and its precursor *in vivo*. Anti-plastocyanin antibodies also immunoprecipitated two polypeptides with apparent molecular weights larger than that of the mature protein (Fig. 3). The largest of these polypeptides has an apparent $M_r$ of 17,000 and comigrates with pre-apoplastocyanin synthesized *in vitro* (17). The other polypeptide migrated with an apparent $M_r$ of 11,000, intermediate

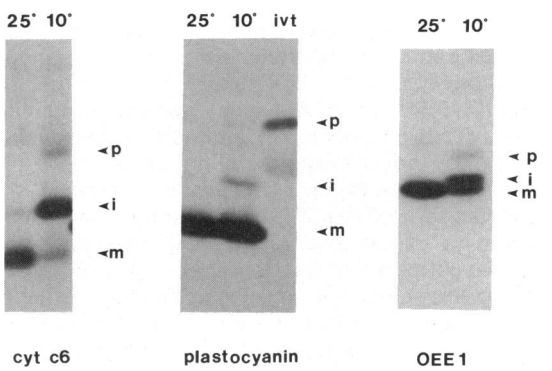


FIG. 3. Effect of reduced temperature on the synthesis of thylakoid lumen proteins. Cells grown in copper-deficient medium were pulse-labeled with $Na_2{}^{35}SO_4$ for 10 min at either 25°C or 10°C and then extracted into acetone. The effectiveness of labeling at 25°C and 10°C was 0.3 and 0.4 trichloroacetic acid-precipitable cpm per cell, respectively. Equal volumes of the two solubilized extracts were immunoprecipitated with polyclonal antibodies raised against cyt *c6* (*Left*), plastocyanin (*Center*), or OEE1 (*Right*). Immunoprecipitates were analyzed by SDS/polyacrylamide gel electrophoresis and fluorography. ivt, Products of a cell-free translation reaction programed with *in vitro* transcribed pre-plastocyanin mRNA. p, Precursor; i, intermediate; m, mature.

between those of the precursor and mature forms. Thus, for each of the lumenal proteins examined (cyt *c6*, OEE1, and plastocyanin), precursor, intermediate, and mature forms are observed during their synthesis *in vivo*. In each case, the short half-lives of the precursor and intermediate species preclude cell fractionation for their suborganellar localization.

The central feature of the two-step model of lumenal protein maturation (4) that remains unproven at this time is that the species with the intermediate electrophoretic mobility is a true intermediate in the biosynthetic pathway—i.e., that it is a substrate for the second translocation and attending processing step. One way to verify this *in vivo* is to show that the intermediate can be quantitatively converted to the mature protein. To facilitate this experiment we sought conditions that would reversibly block the putative "second" processing step and hence result in the accumulation of the intermediate. Since the second of two processing steps in the pathway for mitochondrial cytochrome *c1* maturation is inhibited at reduced temperature in *Neurospora crassa* (20, 21), the effect of low temperature on cyt *c6* synthesis was tested. *C. reinhardtii* cultures cooled to 10°C prior to labeling, in fact, readily accumulate significant amounts of the cyt *c6* intermediate during a 10-min pulse label (Fig. 3). The parallel decrease in the accumulation of the mature protein at 10°C relative to 25°C suggests that reduced temperature does indeed inhibit the processing of the intermediate, albeit not completely (some mature protein was labeled). The effect of reduced temperature was most drastic for cyt *c6* synthesis. The processing of OEE1 also appeared to be slowed at 10°C relative to 25°C, but not to the extent observed for cyt *c6*, and for plastocyanin only a slight increase in the accumulation of the plastocyanin intermediate was observed at the lower temperature.

The accumulation of the putative intermediate form of cyt *c6* at the reduced temperature enabled us to test directly whether this intermediate species was competent as a substrate for processing to the mature form *in vivo*. Cells were labeled with $Na_2{}^{35}SO_4$ for 5 min at 10°C to accumulate the intermediate form and then incubated (chased) with unlabeled sulfate and cycloheximide for 2 min at 10°C. These chase conditions completely inhibit *de novo* cytoplasmic protein synthesis (not shown) and the 2-min incubation allows labeled (full-length) precursor cyt *c6* to turn over (Fig. 4, lane 1). Hence, any labeled *mature* protein that appeared during the subsequent chase period could have come only from the previously synthesized cyt *c6* intermediate. The temperature of the reaction was then either raised to 25°C (Fig. 4, lanes 2 and 3) or maintained at 10°C (lanes 4 and 5) and samples were removed at different times thereafter for analysis. The shift in temperature from 10°C to 25°C caused the accumulated intermediate to be converted to the mature form very rapidly (the estimated $t_{1/2}$ of the intermediate at 25°C was 0.5 min). In cells maintained at 10°C during the chase, the intermediate was also quantitatively converted to the mature form but the reaction was much slower (estimated $t_{1/2}$ at 10°C of 4.5 min). The label originally present in the intermediate was completely recovered in the mature form at 25°C and 10°C (Fig. 4*B*). Furthermore, the mature form of cyt *c6* that appeared during the chase period at either temperature was stable during prolonged chase periods (60 min; not shown), indicating that the mature protein was holocyt *c6* rather than apocyt *c6*. In summary, the intermediate form observed during the biosynthesis of cyt *c6* is competent as a substrate for a second processing step (that is temperature sensitive *in vivo*).

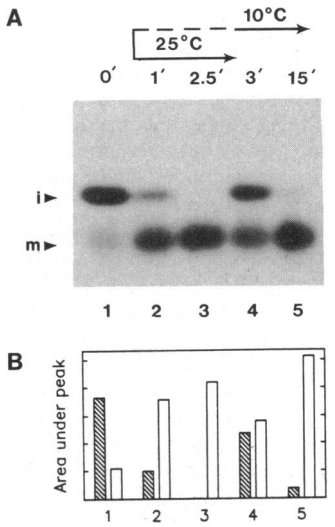


FIG. 4. Precursor–product relationship between the intermediate and mature forms of cyt *c6*. Cells were labeled with $Na_2{}^{35}SO_4$ for 5 min at 10°C and then chased for 2 min at 10°C in the presence of $Na_2SO_4$ and cycloheximide. Samples were then removed into acetone either immediately (0 min time point, lane 1) or after an additional incubation at 10°C for 3 min (lane 4) or 15 min (lane 5). Alternatively, a portion of the 0-min cells (lane 1) was transferred from 10°C to 25°C and samples were then removed into acetone after a continued incubation at 25°C for 1 min (lane 2) or 2.5 min (lane 3). (*A*) Immunoprecipitates were separated by SDS/polyacrylamide gel electrophoresis and the intermediate (i) and mature (m) forms of cyt *c6* were visualized by fluorography. (*B*) The fluorograph in *A* was scanned by laser densitometry. The amount of intermediate cyt *c6* (hatched bar) and mature cyt *c6* (open bar) was quantitated from the absolute area (in arbitrary units) under each peak. The conservation of the label during the experiment was shown by the fact that the sum of the peak areas (intermediate plus mature) in each of the chase samples (lanes 2–5) differed by <15% from each other or from that of the sample at the starting time point (lane 1).

## DISCUSSION

We have used pulse-radiolabeling experiments with *C. reinhardtii* to identify short-lived intermediates in the maturation pathway of the thylakoid lumen protein cyt *c6 in vivo*. Using this approach we detected the synthesis of two polypeptides that we propose are genuine intermediates in this processing pathway. The largest of these polypeptides ($M_r$ 15,000) is identical in electrophoretic behavior to pre-apocyt *c6* synthesized *in vitro* (Fig. 1*B*). [Owing to the limited resolution of the gel system we cannot exclude the possibility that this polypeptide is a slightly altered form of the full-length precursor (e.g., without the initiator methionine).] This precursor, as expected on the basis of our inability to detect it by methods less sensitive than pulse radiolabeling (e.g., Western blot analysis), is subject to rapid turnover *in vivo*. In fact, we estimate that the half-life of full-length pre-apocyt *c6* is comparable to, or shorter than, that of the intermediate (which has a $t_{1/2}$ of ≈0.5 min). Analogous precursor forms of two other lumenal proteins, OEE1 and plastocyanin (Figs. 2*B* and 3), are also detected *in vivo*. Their conversion to the mature protein in earlier *in organello* studies suggested that they may be substrates for the intracellular targeting pathway. Our present results demonstrating these full-length precursors for three different proteins *in vivo* directly confirm the *in organello* work and substantiate that full-length precursor proteins are true *in vivo* substrates in the maturation pathway of lumenal proteins. The model that translocation of precursors into the chloroplast occurs post-translationally is thus extended to an *in vivo* system.

In addition to the identification of the full-length pre-apocyt *c6 in vivo*, we have also detected a polypeptide that is apparently a partially processed (intermediate) form of the preprotein. To determine whether this partially processed polypeptide is a genuine intermediate in the maturation

pathway of cyt *c6*, we used pulse–chase experiments to follow the fate of the molecule *in vivo*. As a consequence of an ≈9-fold decrease in the turnover rate of the intermediate in cells maintained at 10°C relative to cells maintained at 25°C (Fig. 4), the intermediate species accumulated and was demonstrably and quantitatively converted to the mature protein, either upon prolonged incubation at 10°C or upon increasing the temperature to 25°C. This finding provides conclusive experimental evidence that the intermediate form of a lumenal protein is competent as a substrate for processing to the mature protein *in vivo* and thus substantiates the "two-step" model of lumenal protein biosynthesis (4). That the conversion of the cyt *c6* intermediate to its mature form was complete shows that the entire pool of intermediate accumulated at low temperature is competent as a substrate for subsequent maturation and not just a dead-end product deriving from the low-temperature incubation. The step in maturation (intermediate apo- to mature holo-) that is sensitive to low temperature (e.g., translocation across the thylakoid membrane, heme attachment, processing, etc.) is yet to be defined. It is interesting to note that the maturation of plastocyanin, a protein that is functionally equivalent and physically similar to cyt *c6*, is very efficient at low temperature (only a slight increase in the accumulation of the plastocyanin intermediate was observed at low temperature compared to 25°C; Fig. 3). This could indicate that the cold-sensitive step in the maturation of the cyt *c6* intermediate is a reaction that is unique to the biosynthesis of cyt *c6*. For example, the cyt *c6* intermediate may interact with components required for heme attachment (heme or the heme-attaching enzyme) before the processing step occurs (22).

Our identification of intermediate forms of plastocyanin and OEE1 *in vivo* suggests that these proteins, like cyt *c6*, can be processed to their mature forms through an intermediate. Although the *in organello* experiments also included studies of these two proteins, putative intermediates were observed in some cases (1, 4, 6) but not in others (2, 5, 23) and the importance of the intermediate was therefore questioned (6). In no case has it been possible to demonstrate translocation of the intermediate species *in vitro* in the absence of the alternate precursor substrate (5, 7, 8). It is possible that the efficiency of maturation (and hence detection) of intermediates *in vitro* is variable, depending upon the temperature of the assay or whether the precursor and organelle are derived from homologous or heterologous systems. For instance, these factors may influence the conformation of the *in organello*-generated intermediate and hence determine whether the intermediate enters a maturation-competent pool or a pool of dead-end product. Alternatively, the absence of detectable intermediates *in vitro* may indicate that precursor proteins can, under some conditions, be processed to their mature forms in a single step by the thylakoid peptidase (6–9). However, in all cases that we have studied, we detect precursor and intermediate forms *in vivo* and for pre-apocyt *c6* maturation, we have unambiguously established that the intermediate form is indeed a pathway intermediate. Our results for cyt *c6* thus directly and strongly support a two-step import model, and, given the identification of analogous intermediate species for the other two proteins, we propose that two-step processing is a general feature of the targeting of lumenal proteins. Our data do not preclude another intermediate-independent processing pathway that might operate in parallel or as a bypass as is known in mitochondrial systems. For example, the first of the two processing steps that removes the presequence on mitochondrial cytochrome *c1* is not obligatory for the subsequent translocation, heme attachment, and second cleavage (22). The ability to isolate mutants of *C. reinhardtii* that are defective in the biosynthesis of cyt *c6* (12) and to introduce modified genes into the nucleus and chloroplast of this organism should allow us to further dissect the post-translational maturation of chloroplast proteins and determine whether other pathways function *in vivo*. It should also be possible to expand the present study by identifying additional conditions or inhibitors that reversibly block the processing of precursor molecules *in vivo* (e.g., refs. 24 and 25).

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1. James, H. E., Bartling, D., Musgrove, J. E., Kirwin, P. M., Herrmann, R. G. & Robinson, C. (1989) *J. Biol. Chem.* **264**, 19573–19576.
2. Ko, K. & Cashmore, A. R. (1989) *EMBO J.* **8**, 3187–3194.
3. Hageman, J., Baecke, C., Ebskamp, M., Pilon, R., Smeekens, S. & Weisbeek, P. (1990) *Plant Cell* **2**, 479–494.
4. Smeekens, S., Bauerle, C., Hageman, J., Keegstra, K. & Weisbeek, P. (1986) *Cell* **46**, 365–375.
5. Kirwin, P. M., Meadows, J. W., Shackleton, J. B., Musgrove, J. E., Elderfield, P. D., Mould, R., Hay, N. A. & Robinson, C. (1989) *EMBO J.* **8**, 2251–2255.
6. Bauerle, C., Dorl, J. & Keegstra, K. (1991) *J. Biol. Chem.* **266**, 5884–5890.
7. Bauerle, C. & Keegstra, K. (1991) *J. Biol. Chem.* **266**, 5876–5883.
8. Mould, R. M., Shackleton, J. B. & Robinson, C. (1991) *J. Biol. Chem.* **266**, 17286–17289.
9. Klosgen, R. B., Brock, I. W., Herrmann, R. G. & Robinson, C. (1992) *Plant Mol. Biol.* **18**, 1031–1034.
10. Cline, K., Ettinger, W. F. & Theg, S. M. (1992) *J. Biol. Chem.* **267**, 2688–2696.
11. Merchant, S. & Bogorad, L. (1987) *J. Biol. Chem.* **262**, 9062–9067.
12. Howe, G. & Merchant, S. (1992) *EMBO J.* **11**, 2789–2801.
13. Wood, P. (1978) *Eur. J. Biochem.* **87**, 9–19.
14. Hill, K. & Merchant, S. (1992) *Plant Physiol.* **100**, 319–326.
15. Merchant, S. & Bogorad, L. (1986) *J. Biol. Chem.* **261**, 15850–15853.
16. Schmidt, G. W. & Mishkind, M. L. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2632–2636.
17. Merchant, S., Hill, K., Kim, J. H., Thompson, J., Zaitlin, D. & Bogorad, L. (1990) *J. Biol. Chem.* **265**, 12372–12379.
18. Dobberstein, B., Blobel, G. & Chua, N.-H. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1082–1085.
19. Mayfield, S. P., Schirmer-Rahire, M., Frank, G., Zuber, H. & Rochaix, J.-D. (1989) *Plant Mol. Biol.* **12**, 683–693.
20. Teintze, M., Slaughter, M., Weiss, H. & Neupert, W. (1982) *J. Biol. Chem.* **257**, 10364–10371.
21. Schleyer, M. & Neupert, W. (1985) *Cell* **43**, 339–350.
22. Nicholson, D. W., Stuart, R. A. & Neupert, W. (1989) *J. Biol. Chem.* **264**, 10156–10168.
23. Vorst, O., Oosterhoff-Teertstra, R., Vankan, R., Smeekens, S. & Weisbeek, P. (1988) *Gene* **65**, 56–69.
24. Reid, G. A. & Schatz, G. (1982) *J. Biol. Chem.* **257**, 13056–13061.
25. Reid, G. A. & Schatz, G. (1982) *J. Biol. Chem.* **257**, 13062–13067.