Loss of Efficient Import and Thylakoid Insertion Due to N- and C-Terminal Deletions in the Light-Harvesting Chlorophyll a/b Binding Protein

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C-terminally truncated precursors of wheat light-harvesting chlorophyll a/b binding protein (LHCP) were synthesized to investigate the origin of the two forms (about 25 kD and 26 kD) of the mature protein observed upon in vitro import into the chloroplast. Precursors pA13 and pA27, lacking 13 and 27 amino acids, respectively, were successfully imported, and both gave rise to two smaller forms proportional to the size of their deletions. These results demonstrate that there are two N-terminal sites that are cleaved during import of the LHCP precursor, undoubtedly contributing to the heterogeneity of LHCP found in vivo. Significantly, pA27 yielded only 50% of mature LHCP when compared with wild type. Although the products of pA27 import were localized to the thylakoids, in contrast to pA13 they were not correctly inserted into the membranes, indicating that residues essential for this step are missing. pA27 is distinguished from pA13 by lacking the carboxy end of a domain highly conserved between LHCP of photosystems II and I. Other specific precursor mutants with larger C-terminal deletions were not efficiently transported into the organelle in time course experiments, nor did they insert directly into the thylakoids using chloroplast lysates, in an assay independent of translocation across the envelope. In addition, the mutant pA18n, lacking the first 18 amino acids of mature LHCP, was only found bound to the chloroplast envelope. However, both pA18n and the mature protein, i.e., LHCP, synthesized in vitro without its 34-amino acid transit peptide inserted into the thylakoids in chloroplast lysates. The overall conformation of the mutant precursor polypeptides was probed using the chloroplast soluble processing enzyme in an organelle-free reaction optimized for the LHCP precursor and the more general protease trypsin. A tightly folded, protease-resistant conformation was not apparent to explain the loss of efficient import.

INTRODUCTION

The major light-harvesting chlorophyll a/b binding protein associated with photosystem II (LHCP) is encoded by a nuclear multigene family in higher plants (Coruzzi et al., 1983; Dunsmuir, 1985; Lamppa, Morelli, and Chua, 1985; Leutwiler, Meyerowitz, and Towbin, 1986). It is synthesized as a precursor (pLHCP) in the cytoplasm with an N-terminal extension of 34 to 37 amino acids (Cashmore, 1984; Lamppa et al., 1985; Dunsmuir, 1985; Kohorn et al., 1986), which, upon post-translational import into the chloroplast, is processed to its mature form (Schmidt et al., 1981). The two most abundant forms of LHCP have apparent molecular masses of approximately 25 kD and 26 kD to 27 kD. The origin of these peptides has not been fully explained. Recently, genes have been identified that code for one LHCP precursor variant in petunia (Stayton et al., 1986) and Lemna (Kohorn et al., 1986) that, upon import, also yields multiple forms. Analyses of other LHCP-encoding loci in different higher plants show that they code for structurally nearly identical mature proteins (for review, see Buetow et al., 1988). In vitro import studies have produced increasing evidence that the two major forms of LHCP may be due to proteolytic processing of a single precursor substrate. Multiple forms of LHCP have been obtained during the import of pLHCP into the chloroplast whether the gene originates from wheat (Lamppa and Abad, 1987), Lemna (Kohorn and Tobin, 1986), pea (Cline, 1988), corn (Dietz and Bogorad, 1987), or tomato (Pickersky et al., 1987). In addition, Darr, Somerville, and Arntzen (1986) have isolated monoclonal antibodies that recognize the N terminus of the 26-kD form and not the 25-kD peptide, although other monoclonal antibodies react with domains throughout both of the two proteins.

In the present study we have investigated the nature of the mature forms of LHCP found upon import of the precursor into the chloroplast using C-terminally truncated precursors. We wanted to determine whether these mu-
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Residues Deleted

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Figure 1. Description of the pLHCP Deletion Mutants.

The proteins coded for by the wild-type precursor and mutant polypeptides are shown schematically (left), where the transit peptide (T), mature protein, and the residues deleted are indicated (right). The asterisk for the mutant pA48* indicates that besides the deletion, the C-terminal amino acids have been changed (see Methods). The constructs contain the last two codons for the 266-amino acid precursor, including the wild-type termination codon.

RESULTS

Two N-Terminal Processing Events Give Rise to Two Mature Forms during Import of pLHCP into the Chloroplast

pLHCP was synthesized in a reticulocyte lysate using SP6 polymerase transcripts of a wheat gene, previously characterized (Lamppa et al., 1985). Upon incubation of radiolabeled pLHCP with chloroplasts in an in vitro import reaction, two mature forms with apparent molecular mass of about 25 kD and 26 kD are found inserted into the thylakoids, and, as discussed previously, are both generated from the predominant 31-kD precursor. This is the case whether the chloroplasts originate from pea or wheat. In addition, time course studies of import showed that the 26-kD peptide arises before the 25-kD form, and, thus, the 26-kD peptide was most likely not a derivative of the latter because of a covalent modification (Lamppa and Abad, 1987). To investigate further the origin of the two mature forms of LHCP, mutant precursor polypeptides with C-terminal truncations were synthesized. Three truncated precursors (see Figure 1) were initially analyzed in which 13 amino acids (residues 252 to 264, numbering from the N terminus of the 266-amino acid precursor), 27 amino acids (residues 238 to 264), and 91 amino acids (residues 174 to 264) were deleted to produce the mutants pΔ13, pΔ27, and pΔ91 lacking about 1.5 kD, 2.9 kD, and 9.7 kD, respectively.
kD, respectively. The rationale was that if the 34-amino acid transit peptide were removed, giving rise to one form, and a secondary cleavage also occurred at the N terminus, then the two processed forms should exhibit a shift in mobility proportional to the size of the C-terminal deletion when analyzed by SDS-PAGE. Alternatively, if C-terminal processing of a subpopulation of pLHCP removes an additional approximately 1 kD from the mature protein, pΔ13, pΔ27, and pΔ91 should each yield only one peptide upon import because their truncations would result in the loss of the C-terminal cleavage site. In addition, a complementary mutant precursor, pΔ18n, was constructed that lacks the first 18 amino acids of mature LHCP. We predicted that this mutant may not be cleaved by the chloroplast processing enzyme, i.e., transit peptidase, during import because of modification at the junction of the transit peptide and mature protein. However, it should still be recognized by a C-terminal-specific processing enzyme, and, hence, serve as a positive control to identify this activity.

As shown in Figure 2A, both pΔ13 and pΔ27 are imported into the chloroplast. In both instances, two mature forms of LHCP are found in the membrane fraction isolated from organelles that had been treated with thermolysin before lysis. Furthermore, the two mature forms show a shift in mobility during SDS-PAGE, reflecting the size of the C-terminal deletion. These results demonstrate that the lower mature form is not due to a second, C-terminal cleavage, and, thus, two N-terminal processing events occur during import of pLHCP into the chloroplast.

Unexpectedly, when pΔ91 was used in an import reaction, no mature protein was found within the chloroplast, nor was any precursor bound to the envelope. Similarly, after incubating pΔ18n with isolated chloroplasts for 1 hr, no protein was identified within the organelle (Figure 2A). The pΔ18n associated with the membrane fraction was sensitive to treatment of the organelles with thermolysin. This observation suggested that, although pΔ18n was targeted to the chloroplast and was able to bind to the envelope, the mutant precursor was not translocated across the membrane. An alternative explanation was that pΔ18n was imported and rapidly turned over within the organelle because of the N-terminal deletion. To try to distinguish between these possibilities, time courses of pΔ18n and pLHCP import were performed. Figure 2B shows the products associated with the membrane fraction of chloroplasts before and after treatment with thermolysin following reactions carried out for 1 min, 5 min, and 10 min. For pΔ18n, no radiolabeled protein was found in the thermolysin-resistant membrane fraction even at the earliest timepoint, nor in separate reactions was protein identified in the soluble fraction (not shown). Thus, although pΔ18n will bind to the chloroplast, these studies suggest that it is incompetent for translocation into the organelle.

**Products of pΔ27 Import Are not Inserted into the Thylakoids**

To determine whether the mature forms of LHCP produced during import of pΔ13 and pΔ27 were inserted into the thylakoids, the membranes were separated from the stromal fraction and treated with trypsin for 15 min. When LHCP is correctly inserted into the thylakoids, trypsin selectively removes only about 1.5 kD from its N terminus,
The import of pA13 into the chloroplast occurred at the end of C-terminal Deletions. Loss of Efficient Import into the Chloroplast Because of amino acids are the same (Hoffman et al., 1987). To examine further how transport into the chloroplast. To examine further how few as 27 amino acids removed from the C terminus of the mature protein impaired insertion of LHCP into the thylakoid membranes. The amino acids missing in pA27 but retained in pA13 are LGDLADHIPVNN, and they are located at the very end of a domain (residues 191 to 255) highly conserved between LHCP of photosystem II (PSII) and photosystem I (PSI). In fact, 12 of the 14 amino acids are the same (Hoffman et al., 1987).

Loss of Efficient Import into the Chloroplast Because of C-Terminal Deletions

The import of pA13 into the chloroplast occurred at the same efficiency as the wild-type precursor; however, we routinely observed that pA27 yielded less than 50% of the amount of mature protein obtained with the wild-type precursor, and no evidence has been obtained for pA91 transport into the chloroplast. To examine further how deletion of the C terminus of pLHCP affects the efficiency of import, or stable thylakoid insertion, two additional C-terminal mutant proteins were synthesized with intermediate-sized deletions (see Figure 1). Constructs were made coding for the precursors pA53 and pA78 lacking 53 (residues 186 to 239) and 78 (residues 187 to 264) amino acids, respectively. pA53 retains the 27 amino acids absent in pA27, but it is missing residues 186 to 239, almost the entire domain (residues 191 to 255) found conserved between the LHCP polypeptides of PSII and PSI (Hoffman et al., 1987). A third mutant, pA48*, was also designed that lacks the same internal amino acids as pA53 but contains a modified C-terminal sequence of 31 amino acids that is different from the 27 amino acids in the wild-type precursor. The overall charge of this domain was changed from acidic to basic (see Methods).

When pA53, pA78, and pA48*, synthesized in a reticulocyte lysate, were examined for their import competence in a standard 1-hr reaction, no mature protein was found associated with either the membrane or soluble fractions of the organelle, as shown in Figure 4. In each case, a low level of precursor was in the membrane fraction, but this was susceptible to digestion with thermolysin, indicating that it was outside of the organelle, bound to the envelope. To establish whether these mutant precursors entered the organelle and were rapidly degraded, which has recently been observed for a C-terminal deletion of Lemna pLHCP (Kohorn and Tobin, 1989), import reactions were stopped for membrane analysis after 5 min, 10 min, and 20 min. Whereas mature LHCP accumulated in the thermolysin-resistant membrane fraction at the earliest timepoint when the wild-type precursor was assayed, the products associated with the chloroplast using pA53 and pA78 were thermolysin-sensitive, indicating that they had not entered the organelle, as shown in Figure 5. We pursued this point further and analyzed both the membrane and soluble fractions from import reactions. Although pA53 binds to the chloroplast, radiolabeled protein was not found within the organelle in either the membrane or soluble phases from reactions stopped at 1 min, 5 min, and 10 min. Similar results were obtained for pA78 (data not shown). Two low molecular weight products were found in both the membrane and soluble phases after a reaction using pA53, but they were not present in the thermolysin-treated samples. To confirm that the disappearance of the two products was due to thermolysin sensitivity and not turnover of imported protein during the treatment, in a separate experiment, one-half of an import reaction was diluted fourfold and held on ice for an additional 15 min, whereas the other half was treated with thermolysin, instead of immediately lysing after a 5-min import reaction. As shown in Figure 6, the two products were still present in the untreated soluble fraction at the end of 15 min, but were again absent in the thermolysin-treated sample.

Identical reactions were also performed for pLHCP and pA78. Whereas mature LHCP was found in the thermolysin-sensitive approximately 24-kD peptide fragment (Figure 3; see also Mullet, 1983). Although some of the 25-kD peptide is still present after trypsin treatment, our results indicate that this is due to incomplete digestion because after longer digestions, only the 24-kD fragment is found. Digestion of the membrane fraction with trypsin after import of pA13 also produces the expected resistant peptide. In contrast, in a parallel reaction, no trypsin resistant peptides were found after import of pA27. Thus, although the truncated protein localized to the correct suborganellar compartment, as few as 27 amino acids removed from the C terminus of the mature protein impaired insertion of LHCP into the thylakoid membranes. The amino acids missing in pA27 but retained in pA13 are LGDLADHIPVNN, and they are located at the very end of a domain (residues 191 to 255) highly conserved between LHCP of photosystem II (PSII) and photosystem I (PSI). In fact, 12 of the 14 amino acids are the same (Hoffman et al., 1987).
Figure 4. Import Reactions for the C-Terminal Mutants pA48*, pA53, and pA78.

The mutant precursors were incubated with chloroplasts for 1 hr, and the membrane and soluble fractions were isolated from thermolysin-treated and nontreated organelles and analyzed by SDS-PAGE. Lanes are as described in Figure 2. Wild-type (WT) precursor was analyzed in parallel to demonstrate import competence of chloroplasts.

Analysis of Thylakoid Insertion Using a Chloroplast Lysate Assay

Using a hybrid precursor composed of the transit peptide of the S precursor (pS) and mature LHCP, we have demonstrated that the cognate transit peptide of LHCP is not required for thylakoid insertion (Lamppa, 1988). We had initially anticipated that the C terminus mutants described here would provide a clue as to the signal in mature LHCP that localizes it to the thylakoids. Because these mutants contain an intact transit peptide, it was somewhat unexpected that they were not found within the organelle. To address the question of thylakoid localization in the absence of import into the chloroplast, we have employed a chloroplast lysate assay using conditions as described by Cline (1988). The mutants pA16*, pA48*, and pA53, in parallel with the wild-type precursor, were incubated with lysed chloroplasts containing both the membrane and soluble phases. As shown in Figure 7, the wild-type precursor localized to the thylakoids, and, upon treatment with trypsin, gave rise to an approximately 23-kD to 24-kD trypsin-resistant membrane fraction using the wild-type precursor, pA78 did not give rise to any thermolysin-resistant protein in either the membrane or soluble fractions. In previous experiments, we have demonstrated that proteins localized to the stroma after import, such as the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (S), are resistant to the thermolysin conditions used in these experiments (Lamppa, 1988). Taken together, these results indicate that the two low molecular weight products of the pA53 import reaction are on the exterior of the organelle, and that pA53 and pA78 are not efficiently transported into the chloroplast.

Figure 5. Time Course of pA53 and pA78 Import.

Analysis of membrane proteins after a reaction from thermolysin-treated (M+Th, lanes 3, 5, and 7) and nontreated (M, lanes 2, 4, and 6) chloroplasts. The reactions were stopped at 5 min, 10 min, and 20 min and the nonthermolysin-treated samples were immediately lysed. WT, wild type.
resistant product, indicating that it was correctly inserted with only its N terminus accessible to cleavage. A very small amount of pΔ18n also integrated into the membranes despite its N-terminal deletion. In contrast, although some of both pΔ48* and pΔ53 were found associated with the thylakoids, neither was resistant to protease treatment. This was also true for pΔ27 in this assay (not shown) as well as during import into chloroplasts (Figure 3). Although pΔ53 contains the 27 amino acids missing in pΔ27, it was not able to insert into the thylakoids. Therefore, it is not a specific sequence within the last 27 amino acids (residues 238 to 264) of mature LHCP alone that directs thylakoid insertion, nor is the remainder of the protein sufficient for this event because pΔ27 did not insert.

To establish that the defect resulting in a loss of thylakoid insertion was indeed the result of disruption of important features in the mature portion of the precursor and not due to a masking of the transit peptide as a result of the deletion, the mutant protein Δ34t was synthesized from transcripts lacking the sequence coding for the 34-amino acid transit peptide of wheat pLHCP. Both pLHCP and Δ34t were used in an insertion assay. As shown in Figure 8A, incubation of pLHCP with chloroplast lysates again resulted in the association of the precursor with the membranes and some processing to the mature forms (left, lane 2). The mutant Δ34t was also found in the membrane fraction (right, lane 2) and, upon trypsin treatment, yielded the same approximately 23-kD to 24-kD resistant peptide as the wild-type precursor (Figure 8A, see asterisks, lanes 3).

Analysis of Precursor Sensitivity to Protease Digestion

The structural changes in the mutant precursors of LHCP were sufficient to prevent both efficient import and thylakoid insertion. We attempted to determine how the conformation of the mutant precursors may have been changed by examining their relative sensitivity to proteolytic cleavage using two different enzymes. We addressed the question of whether the mutant substrates would be recognized by the chloroplast soluble processing enzyme that we have recently described for pLHCP cleavage in an organelle-free reaction (Lamppa and Abad, 1987; Abad, Clark, and Lamppa, 1989). The properties of the soluble enzyme ($M_r = 240,000$) are similar to those described for the enzyme that removes the transit peptide of ps and pre-plastocyanin (Robinson and Ellis, 1984). In this reaction, pLHCP is cleaved, releasing a 25-kD peptide that migrates with the lower mature form of LHCP obtained during import. Both pΔ13 and pΔ27 were processed in this assay, releasing proportionally smaller peptides, demonstrating that N-terminal cleavage indeed occurs. On the other hand, pΔ91 was not processed (Abad et al., 1989).

In addition to probing the overall structural changes of the mutant precursors with this large protease that cleaves at a specific N-terminal site, we have also performed a time course of precursor digestion by trypsin ($M_r = 23,500$), which will cut throughout the polypeptide at arginine and lysine residues. Our rationale for these experiments was based on the following. First, destabilization of the tertiary structure of dihydrofolate reductase enhances transport into the mitochondria when fused to the presequence of cytochrome oxidase. That is, an "unfolded" precursor state, as assessed by an increased susceptibility to trypsin digestion, is required for rapid import (Vestweber and...
The results of organelle-free processing reactions for the wild-type precursor and pΔ18n, pΔ48*, pΔ53, and pΔ78 are shown in Figure 9. The C terminus mutants pΔ48* and pΔ53 were processed by the chloroplast enzyme as efficiently as wild type, pΔ78 was cleaved but somewhat less efficiently, and pΔ18n was not processed at all. The fact that the mutation of pΔ18n occurs at the junction of the transit peptide mature protein may be the determining factor for loss of processing, despite the presence of an intact transit peptide. On the other hand, the structural changes conferred by the C-terminal deletions either are apparently overcome by interaction with the processing enzyme or they do not alter the conformational features of the precursor essential for cleavage in the organelle-free reaction. It is important to emphasize, however, that, in addition to pΔ18n, we have not observed processing of pΔ91 (Abad et al., 1989), which lacks two-thirds of the mature protein.

Along with the wild-type precursor, the mutants pΔ53 and pΔ78 were also examined for their relative sensitivity to trypsin. There are 21 trypsin-sensitive sites in the wild-type precursor and 15 and 16 in pΔ78 and pΔ53, respectively. Time courses were performed at a trypsin concentration of 1 mM. As shown in Figure 10, all three precursors were equally sensitive to the protease, disappearing with similar kinetics. Most importantly, no major resistant peptides were identifiable upon prolonged treatment, as would be expected if the truncated polypeptides were tightly folded, or aggregated, thereby protecting internal arginine

Schatz, 1988). Second, efficient export of the maltose-binding protein in Escherichia coli depends on the product of the SecB gene, which, based on proteinase K sensitivity studies, maintains an unfolded precursor structure (Collier, Bankaitis, and Bassford, 1988; Kumamoto and Gannon, 1988). Thus, we examined the possibility that the C-terminal deletions of the mature portion of pLHCP may have altered precursor folding, producing a protease-resistant conformation.

Figure 8. Analysis of Mature LHCP Insertion into the Thylakoids Using the Mutant Δ34t Lacking the Transit Peptide.

(A) Thylakoid insertion assays were performed using both the wild-type (WT) precursor and Δ34t translation products (TP, lanes 1). Protein associated with the thylakoids before (M, lanes 2) and after (M+Tr, lanes 3) trypsin treatment are shown. Estimated protein sizes (kD) are given at the left.

(B) Results of an insertion assay using the wild-type (WT) precursor and the products of a translation reaction to which no RNA was added. As indicated by the small arrows, the reticulocyte translation mixture produced significant amounts of radiolabeled aminoacyl-tRNA that was removable by RNase digestion (not shown). In each panel the trypsin-resistant protein is marked by an asterisk.

Figure 9. Analysis of Precursor Sensitivity to the Chloroplast Processing Enzyme.

Organelle-free processing of the precursor mutants using a soluble chloroplast extract. The products of a processing reaction are shown for the wild-type precursor (lane 1), pΔ18n (lane 2), pΔ48* (lane 3), pΔ53 (lane 4), and pΔ78 (lane 5, short autoradiograph exposure; lane 6, extended exposure).
these changes, especially reflected in the peripheral components of the light-harvesting complex II (LHCII). The two peptides change in their relative amount and distribution between the grana and stromal thylakoids during pea development and in response to different light regimes (Larsson et al., 1987a, 1987b). It has been proposed that these changes, especially reflected in the peripheral component of LHCII, provide an adaptive strategy to optimize photosynthetic efficiency as well as to protect the photosystems from high-intensity light (Anderson and Andersson, 1988).

To establish that the two mature forms of LHCP were both due to N-terminal cleavage, we initially employed three C-terminally deleted precursors, pΔ13, pΔ27, and pΔ91. The findings that pΔ27 yielded only 50% mature LHCP as compared with the wild-type precursor and that pΔ91 appeared not to be imported were unexpected, and, consequently, prompted the synthesis of three additional deletion mutants, pΔ53, pΔ78, and pΔ48*, to refine our analysis. Despite intact transit peptides and the fact that the deletions begin 154 amino acids away, no evidence has been found for the transport of these mutant precursors into the chloroplast. If a low level of import does occur, then precursor degradation must occur almost concomitantly. Because the N-terminal mutant pΔ18n was not found within the organelle either, it seems unlikely that it is the loss of a specific C-terminal sequence of LHCP that results in a loss of import function.

The mutants pΔ53, pΔ48*, and pΔ18n were analyzed for their ability to insert into the thylakoids in the absence of import, using a chloroplast lysate assay (Cline, 1988). pΔ53 and pΔ48* did not insert into the thylakoids under conditions in which the wild-type precursor does. In contrast, we have synthesized wheat mature LHCP Δ34t, without a transit peptide, and find that it inserts into the thylakoids, confirming both the study of Viitanen, Doran, and Dunsmaur (1988) and our earlier results (Lamppa, 1988) using wheat LHCP fused to the transit peptide of pS, which show that the mature protein contains the signal for thylakoid localization upon import. Because pΔ18n also was capable of thylakoid insertion, albeit less efficiently, it appears that the N terminus of LHCP is not essential for this step.

A model for LHCP insertion into the thylakoids has been presented that includes three α-helical membrane-spanning domains, with the N terminus of the protein extending into the stroma and the C terminus into the lumen (Karlin-Neumann et al., 1985). Aspects of this model have recently been tested, and it has been shown that a mutant with a 15-amino acid deletion of the third membrane-spanning domain, called helix 3, is imported but does not stably insert into the thylakoids. However, helix 3 itself will not insert into the thylakoids if residues 119 to 144 of LHCP are deleted (Kohorn et al., 1986; Kohorn and Tobin, 1989). Except for pΔ13 and pΔ27, the C-terminal deletions described in our study begin beyond the first two potential membrane-spanning domains and they are missing helix 3. Although pΔ13 is imported and inserted into the thylakoids, we find that pΔ27 localizes to the thylakoids but remains only peripherally associated. While retaining helix 3, pΔ27 is minus an acidic and polar domain predicted to extend into the lumen. It is interesting to speculate that this domain anchors the mature protein into the thylakoids after correct intraorganellar localization has occurred, and that this step occurs through an interaction with helix 3. Significantly, pΔ53 will not insert into the thylakoids although it contains the C-terminal 27 amino acids absent in pΔ27; thus, these residues do not contain an independent signal for insertion. It is also of interest to note that, with
the exception of pΔ13, the C-terminal deletions all fall within a region of the protein that is highly conserved between LHCP of PSII and PSI (Hoffman et al., 1987). Our results indicate that this conserved region indeed plays an important functional role in the association and insertion of LHCP into the thylakoids.

Although pΔ27 did not insert into the thylakoids, it was proteolytically processed to two mature forms. Thus, these two steps in the LHCP import pathway are not obligatorily coupled, and processing can occur without the structural changes that most likely occur concomitant with membrane insertion. We have also shown recently that a 4-amino acid insertion at the transit peptide-mature protein junction that blocks processing does not prevent thylakoid insertion of a mutant called pI+4 (Clark et al., 1989). Chitnis et al. (1988) have also observed, using etioplasts with an inactive processing enzyme, that pLHCP inserts into the thylakoids. The temporal order of the processing and insertion steps in vivo has not yet been established, but it appears that a defect in one step does not necessarily disrupt the other.

The tertiary structure of the mutant precursors was probed by determining their relative sensitivity to protease digestion. Proteinase K and trypsin, for example, have been used to discriminate between tightly folded states of dihydrofolate reductase fused to a mitochondrial presequence that make it incompetent for import into mitochondria, and unfolded conformations that promote its translocation efficiency (Eilers and Schatz, 1986; Eilers, Hwang, and Schatz, 1988; Vestweber and Schatz, 1988). Here, the mutant LHCP precursors were first analyzed for their sensitivity to the chloroplast processing enzyme in an organelle-free reaction, optimized for pLHCP (Lamppa et al., 1987). Abad et al. (1989). Along with the wild-type precursor, pΔ13, pΔ27, pΔ48*, pΔ53, and pΔ78 were cleaved, producing a single peptide, as is characteristic of this assay. Thus, the features of these mutants that may interfere with efficient import and thylakoid insertion do not inhibit the processing enzyme. Only p18n and pΔ91 were resistant to processing, indicating the severity of their deletions (Figure 9, and see Abad et al., 1989). pΔ18n may be resistant to cleavage because the deletion occurs at the transit peptide-mature protein junction; however, this does not easily explain the loss of import. In other experiments, using the mutant pI+4 described above, import still occurred although processing was blocked because of the mutation in the junction domain (Clark et al., 1989), demonstrating that translocation across the envelope does not depend on processing, an observation also recently made for pS (Reiss, Wasmann, and Bohnert, 1989). The conformational structures of pΔ53 and pΔ78 were also probed with trypsin and compared with wild type, but the patterns of precursor degradation and the disappearance of radiolabeled subfragments were not substantially different. Thus, the deletions do not result in a detectable tight misfolding of these precursors or aggregative formation that would protect internal arginine and lysine residues from cleavage by trypsin. Nor have we found that the wild-type precursor is more resistant to proteolysis because of a unique conformation.

In conclusion, we have shown that, when pLHCP is imported into the chloroplast, it can be processed at two N-terminal sites. Time course experiments indicate that efficient import can be blocked by both N-terminal (e.g., pΔ18n) and C-terminal (e.g., pΔ53) deletions in the mature protein, emphasizing that the transit peptide alone is not sufficient to mobilize LHCP into the chloroplast if the structure of the mature protein is significantly altered. Although major structural changes were not obvious using protease resistance as a criterion, it is still quite possible that the loss of efficient import is due to the fact that the precursor undergoes a major conformational change as a result of each deletion, which could indirectly affect the ability of the transit peptide to mediate entrance into the import pathway or prevent essential features of the mature protein itself from participating in the import process. Mitochondrial import requires hydrolyzable nucleoside triphosphates (Chen and Douglas, 1987; Eilers, Oppliger, and Schatz, 1987; Pfanner, Tropschug, and Neupert, 1987) which, along with hsp70 (Deshaies et al., 1988), may be required for unfolding of precursor proteins, making them translocation competent. Different precursors required different amounts of nucleoside triphosphates, presumably because of their different tertiary structures (Pfanner et al., 1987). Import of proteins into the chloroplast also requires ATP (Grossman, Bartlett, and Chua, 1980; Pain and Blobel, 1998); however, its role or potential to affect precursor conformation during import is not yet defined. Understanding mechanistically how the deletion mutants described in the present report interfere with successful transpor of LHCP into the chloroplast and thylakoid insertion awaits further study.

METHODS

Plant Growth and Chloroplast Isolation

Peas (Pisum sativum, Laxton’s Progress) were grown at 26°C under cool-white fluorescent lights on a 12 hr light/12 hr dark cycle, and harvested 7 days after germination. Plants were homogenized with a Polytron at 4°C in 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 50 mM Hepes-KOH, pH 7.5, 0.33 M sorbitol, 5 mM sodium ascorbate, 0.25% BSA at a ratio of 10 mL of buffer/g of tissue, fresh weight. Procedures described by Bartlett, Grossman, and Chua (1982) were used to purify intact chloroplasts on Percoll gradients.

Plasmid Constructions

A wheat gene coding for pLHCP (Lamppa et al., 1985) was inserted into the transcription vector SP65, producing the plasmid
The SP6 polyermase transcription reactions (Promega-Fisher) and for transformation. The deletion was confirmed by sequence analysis using the dideoxynucleotide chain termination method (Sanger, Nicklen, and Coulsen, 1977). The deletion resulted in the loss of residues 186 to 239 from the 266-amino acid LHCP precursor. The 4.4-kb fragment was isolated and treated with Rsal and Xhol, releasing a 142-bp Rsal-Xhol fragment. The 967-bp fragment was treated with HincII to release a 3.6-kb fragment, containing the two C-terminal amino acids (242 to 266) into the plasmid SP65-LHCP (Lamppa and Abad, 1987). The DNA constructs in SP65 coding for the C-terminal mutants pA13, pA37, and pA91 were prepared as previously described (Abad et al., 1989). To create the constructs coding for pA54, pA55, and pA56, the SalI site was first deleted from the polylinker of the SP65 vector by linearizing with Sall and treating the plasmid with mung bean nuclease (Pharmacia LKB Biotechnology Inc.), producing SP65.sal. The wheat pLHCP gene flanked by Psst sites on a 1.6-kb fragment was then inserted into the Psst site of SP65.sal. SP65.sal-LHCP.A53 was constructed by treating SP65.sal-LHCP with Sall, treating with the Klenow fragment of DNA polymerase I (2 units/μg DNA), and ligated with T4 DNA ligase, and used to transform competent Escherichia coli HB101 cells. The 159-bp deletion resulted in the loss of residues 186 to 239 from the 266-amino acid LHCP precursor. SP65.sal-LHCP.A48 was constructed by linearizing SP65.sal-LHCP with Sall, treating with the Klenow fragment of DNA polymerase I (2 units/μg DNA), and then digesting with ApaI to release both a 4.4-kb and 157-bp fragment. The isolated 4.4-kb fragment was treated with mung bean nuclease, creating blunt ends that were ligated to reclose the plasmid. The 157-bp deletion removed the codons for residues 188 to 241 of the mature protein, creating a shift in the reading frame to IGGPCRPH-IGGPCRPH-IGGPCRPH. The samples were prepared for electrophoresis by solubilizing in loading buffer (0.2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.5 M Tris-HCl, pH 6.8, 0.01% bromophenol blue) and analyzed by SDS-PAGE on 12% or 15% acrylamide gels.

**Thylakoid Insertion Assays**

The procedure was essentially as described by Cline (1988). Chloroplasts (500 μg of chlorophyll/mL) were lysed on ice for 5 min and then the radiolabeled precursor was added to a reaction containing 22 mM Hepes-KOH, pH 8, 0.11 M sorbitol, 10 mM ATP, and 2.5 mM methionine, and carried out for 45 min at 26°C. The samples were then washed with 0.5× HSM, and half of the resuspended membranes was treated with trypsin (100 μg/mL) at 25°C for 15 min, washed with 10-fold excess of 1 mM PMSF, and prepared for electrophoresis, as was the untreated sample.

**Organelle-Free Proteolytic Processing and Trypsin Digestion**

The organelle-free processing reaction was performed as described (Lamppa and Abad, 1987) and optimized (Abad et al., 1989). Basically, 2 μL of radiolabeled precursor was added to a reaction containing 22 mM Hepes-KOH, pH 8, 0.11 M sorbitol, 10 mM ATP, and 2.5 mM methionine, and carried out for 45 min at 26°C. The samples were then washed with 0.5× HSM, and half of the resuspended membranes was treated with trypsin (100 μg/mL) at 25°C for 15 min, washed with 10-fold excess of 1 mM PMSF, and prepared for electrophoresis, as was the untreated sample.

**In Vitro Transcription, Translation, and Import**

The SP6 polymerase transcription reactions (Promega-Fisher) and the reticulocyte translation reactions (Bethesda Research Laboratories) were carried out as recommended to synthesize 35S-methionine-labeled precursor polypeptides. The import assays (350-μL volumes) using radiolabeled precursors and pea chloroplasts, 50 mM Hepes-KOH, pH 7.5, 0.33 M sorbitol, 8 mM methionine (HSM) were presented previously in detail (Lamppa and Abad, 1987; Lamppa, 1988). For time course experiments, the import reaction mixture was immediately placed on ice to stop the reactions at the indicated time, and half of the sample was treated with thermolysin (100 μg/mL) in 2 mM CaCl2 for 45 min to 20 min on ice. Meanwhile, the nontreated part of the sample was immediately diluted threefold with HSM, pelleted, and lysed in 1 mM PMSF on ice. The thermolysin reaction was stopped by a threefold dilution with HSM and the addition of EGTA to 40 mM and PMSF to 2 mM. The chloroplasts were pelleted and then lysed with 1 mM PMSF. Membrane and soluble fractions were separated by centrifugation at 16,000g for 10 min. The protein in the soluble fraction was precipitated in 10% TCA for a minimum of 1 hr at 4°C and washed twice with 80% acetone. The samples were prepared for electrophoresis by solubilizing in loading buffer (0.2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.5 M Tris-HCl, pH 6.8, 0.01% bromophenol blue) and analyzed by SDS-PAGE on 12% or 15% acrylamide gels.

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