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A Hydrophobic, Carboxy-Proximal Region of a Light-Harvesting Chlorophyll *a/b* Protein Is Necessary for Stable Integration into Thylakoid Membranes

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Proteins synthesized as soluble precursors in the cytoplasm of eukaryotic cells often cross organellar membrane barriers and then insert into lipid bilayers. One such polypeptide, the light-harvesting chlorophyll *a/b*-binding protein (LHCP), must also associate with pigment molecules and be assembled into the photosystem II light-harvesting complex in the chloroplast thylakoid membrane. A study of the import of mutant LHCPs into isolated chloroplasts has shown that a putative α-helical membrane-spanning domain near the carboxy terminus (helix 3) is essential for the stable insertion of LHCP in the thylakoid. Protease digestion experiments are consistent with the carboxy terminus of the protein being in the lumen. This report also shows that helix 3, when fused to a soluble protein, can target it to the thylakoids of isolated, intact chloroplasts. Although helix 3 is required for the insertion of LHCP and mutant derivatives into the thylakoid, the full insertion of helix 3 itself requires additionally the presence of other regions of LHCP. Thus, LHCP targeting and integration into thylakoid membranes requires a complex interaction involving a number of different domains of the LHCP polypeptide.

INTRODUCTION

Many proteins are synthesized on free cytoplasmic polysomes as soluble precursors and then become integral membrane proteins in organelles (Verner and Schatz, 1988). It is of interest to determine how such proteins traverse both the cytoplasm and membrane barriers, and then insert into lipid bilayers. The light-harvesting chlorophyll-binding proteins (LHCPs) are integral membrane proteins of the chloroplast that are imported from the cytoplasm as precursors (Schmidt and Mishkind, 1986). LHCP binds several pigment molecules within the chloroplast and assembles into the light-harvesting complex II (LHCII) that transfers absorbed light energy to the photosystem II reaction center (Thornber, 1975; Murphy, 1986).

The amino-terminal transit peptides of chloroplast protein precursors contain necessary, but not always sufficient, information to target the protein to the chloroplast (Keegstra and Bauerle, 1988). Sequences within the mature protein also appear to play a role in directing the protein to its final location within the chloroplast (Kohorn et al., 1986; Smeekens et al., 1986), but the mechanisms used have not been determined. Correct import and localization of mitochondrial precursors may require both specific amino acid signals and an ordered unfolding and refolding of a protein (Pfanner et al., 1988). Investigations with bacterial membrane proteins also point to the involvement of multiple domains that affect the insertion of a protein into the lipid bilayer (Dalbey and Wickner, 1988). This report describes some requirements for the insertion of LHCP into thylakoid membranes.

Radiolabeled precursor LHCP (pLHCP) synthesized in vitro from a cloned gene can be imported into isolated chloroplasts, and pLHCP is processed correctly, inserted into the thylakoid membrane, and combined with pigments to form LHCII (Kohorn et al., 1986; Schmidt and Mishkind, 1986). Amino acid sequences important in either thylakoid insertion of LHCP or LHCII assembly were identified by studying the effects on these processes of various deletions (Kohorn et al., 1986) or specific amino acid substitutions (Kohorn and Tobin, 1987) in the protein. One deletion mutant of pLHCP that lacks 15 amino acids of a carboxy-proximal hydrophobic sequence (helix 3) could not be detected in thylakoids after incubation with isolated, intact chloroplasts (Kohorn et al., 1986). The region that includes these 15 amino acids is extremely well conserved in higher plant LHCPs associated with LHCII (Chitnis and Thornber, 1988). It is shown here that this deletion mutant can be imported into the chloroplast but is unable to associate stably with thylakoid membranes. An analysis of fusion proteins shows that helix 3 is able to target a soluble protein to the thylakoid, yet itself cannot cause complete

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membrane integration. Experiments with another LHCP deletion mutant show that, although helix 3 is required for protein integration, the full insertion of that region itself requires additional regions of the LHCP.

RESULTS

A Model for LHCP in the Membrane

A possible conformation of LHCP in the thylakoid membrane has been predicted from the deduced amino acid sequence (Karlin-Neumann et al., 1985), experimental observations (Steinback et al., 1979), and electron microscope studies (Kuhlbrandt, 1984; Li, 1985). Figure 1, panel I, shows a diagram of that model in which three α-helical portions of LHCP are proposed to form membrane-spanning segments, with the amino terminus within the stroma and the carboxy terminus within the lumen. Panel II shows the results of trypsin and carboxypeptidase A treatments of intact thylakoid membranes that contain newly imported, radiolabeled LHCP. Undigested LHCP migrates as a 28-kD band (lane A) and trypsin is able to cleave only about a 2-kD segment from the amino terminus (lane B) (Kohorn et al., 1986; Steinback et al., 1979). Treatment of thylakoids with carboxypeptidase A shows that inserted LHCP

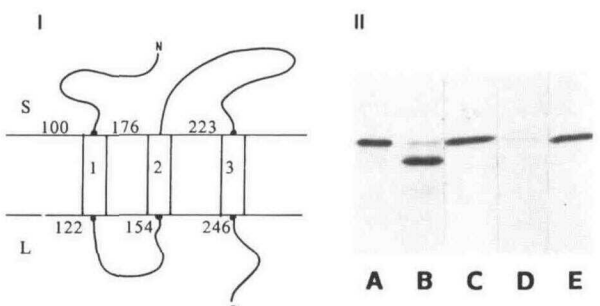

Figure 1. Protease Digestions of LHCP in Thylakoids.

(Panel I) Predicted topography of LHCP in the thylakoid membrane (Karlin-Neumann et al., 1985; Kohorn et al., 1986). N, amino terminus; C, carboxy terminus; S, stroma; L, lumen. Numbers refer either to the amino acid sequence, beginning with the initiating methionine of pLHCP, or to the three putative membrane-spanning helices, 1 to 3.

(Panel II) Radiolabeled pLHCP was imported into isolated pea chloroplasts, and equal aliquots of the thylakoid fraction were subjected to protease digestions, electrophoresis in a denaturing 15% polyacrylamide gel, and fluorography. Lane A, no protease; B, 0.1 mg/ml trypsin; C, 0.1 mg/ml carboxypeptidase A; D, same as in C except with 25 mg/ml Triton X-100; E, same as in D, except with 1 mg/ml carboxypeptidase A inhibitor. Reactions shown in lanes C to E contained 1 mM PMSF to inhibit contaminating protease activity.

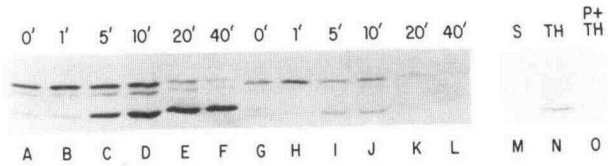

Figure 2. Time Course of Import of pLHCP and a Deletion Mutant J-pLHCP (Kohorn et al., 1986) That Lacks Amino Acids 225 to 240.

Equal amounts of radiolabeled precursor and *Lemna* chloroplasts were incubated, and aliquots of intact plastids were removed from the import reaction at various times, washed in import buffer containing nigericin to inhibit further import (Cline et al., 1985), dissolved, and analyzed by gel electrophoresis and fluorography. Times of import in minutes are indicated above each lane. Lanes A to F represent pLHCP, lanes G to L, J-pLHCP. A similar 10-min J-pLHCP import reaction was terminated and separated into a stromal (S, lane M) and thylakoid (TH, lane N) fraction, and the thylakoids were also treated with 0.1 mg/ml trypsin (P + TH, lane O).

is resistant to this protease (lane C), but inclusion of detergent to solubilize the membrane during protease digestion allows carboxypeptidase to digest most of the labeled LHCP (lane D). As expected, the carboxypeptidase activity is inhibited by carboxypeptidase A inhibitor (lane E), but not by phenylmethylsulfonyl fluoride (PMSF) (lanes C to E). These results are consistent with the placement of the carboxy terminus in the lumen.

Helix 3 Is Required for Stable Insertion

When a labeled deletion mutation of pLHCP, J-pLHCP (Kohorn et al., 1986), that lacks 15 amino acids of helix 3 (amino acids 225 to 240, Figure 1) was incubated with isolated chloroplasts for more than 30 min, no protein could be detected in the chloroplast (Kohorn et al., 1986). To determine whether J-pLHCP is indeed imported into the chloroplast but then degraded rapidly, intact chloroplasts were removed from an import reaction at various times of incubation with labeled protein, washed in import buffer containing nigericin to remove unbound precursor and to inhibit further import (Cline et al., 1985), and analyzed by denaturing electrophoresis and autoradiography. The intact chloroplasts were not treated with thermolysin (Cline et al., 1985), so that protein bound to the surface of the chloroplast could be detected. Figure 2 shows a time course of import for pLHCP and J-pLHCP. For both the wild-type and mutant LHCP, three protein bands are seen at each time point. The two slower migrating forms of each lane are sensitive to thermolysin treatment when this protease is added before the chloroplasts are broken (data not shown), indicating that these proteins are in or on the outer envelope (Cline et al., 1985). These proteins corre-

spond in size to the precursor and a processing intermediate. The smallest protein of each lane corresponds in size to correctly processed LHCP or J-LHCP, and remains after a protease treatment of intact organelles (data not shown). Wild-type processed LHCP accumulates over time (lanes A to F), but processed J-LHCP is degraded within 20 min (lanes G to L).

Chloroplasts from a 10-min incubation of the import reaction were then isolated, lysed, and fractionated to determine whether J-LHCP was associated with the thylakoids or the stroma. LHCP is found exclusively in the thylakoid fraction, and Figure 2 shows that mature J-LHCP also appears in the thylakoids (lane N) and not in the stroma (lane M). When thylakoids containing J-LHCP are digested with trypsin (lane O), no radioactive protein is detected, indicating that J-LHCP is not integrated correctly into the membrane. J-LHCP does not fractionate with the envelope membranes (Kohorn et al., 1986).

Helix 3 Causes Thylakoid Association

To determine whether residues within helix 3, which are required for the stable association of LHCP with the thylakoid, include a thylakoid targeting signal, the import of a fusion peptide containing the small subunit (SSU) of the soluble enzyme ribulose bisphosphate carboxylase fused to the carboxy-terminal region of LHCP containing helix 3 was studied. The fusion protein was synthesized in vitro from a clone that contained LHCP amino acids 210 to 271 (Figure 1) added to the carboxy-terminal amino acid of the entire precursor SSU (pSSU) coding region. This fusion protein, pSSU3LHCP, of 27 kD predicted apparent molecular weight, was expected to be imported into chloroplasts using the pSSU transit peptide, and possibly to target to the thylakoid.

Radiolabeled pSSU3LHCP was incubated with isolated chloroplasts, and the stromal and thylakoid fractions were analyzed by denaturing gel electrophoresis and autoradiography. Figure 3 shows the results of an import of pLHCP, pSSU, and pSSU3LHCP into isolated chloroplasts. LHCP is found as expected in the thylakoid fraction as a 28-kD processed form. The SSU3LHCP fusion protein is also found in the thylakoids, and the predominant form has a size of 23 kD. This is larger than the expected 21-kD processed fusion protein (pSSU transit peptide is about 6 kD), indicating that SSU3LHCP has either an anomalous migration, which is, in fact, seen for LHCP itself (Kohorn et al., 1986), or that processing is incorrect. Less pSSU3LHCP than pLHCP is detected with the thylakoid membranes, yet, significantly, neither LHCP nor SSU3LHCP is detected in the stroma.

To determine which portion of LHCP amino acids 210 to 271 cause the thylakoid association, a second fusion protein, pSSUCLHCP, was constructed and synthesized. pSSUCLHCP contains pSSU and only the carboxy-terminal LHCP amino acids 241 to 271. When pSSUCLHCP is

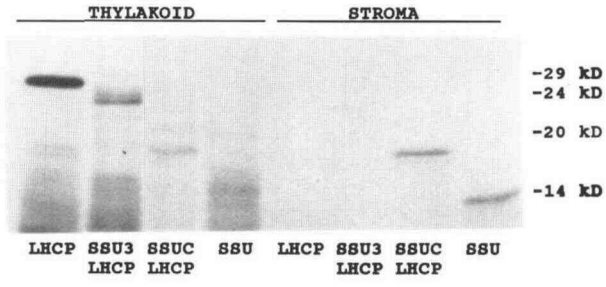

Figure 3. A pSSU-3LHCP Fusion Is Imported and Associates Preferentially with Thylakoids.

Equal amounts of radiolabeled proteins were incubated in an import reaction with isolated *Lemna* chloroplasts for 30 min. Intact chloroplasts were treated with thermolysin and repurified, and then the stromal and thylakoid fractions were isolated. Fractions were analyzed by electrophoresis in a denaturing 10% to 20% polyacrylamide gel and fluorography. Thylakoid and stromal fractions are indicated above lanes. The precursors imported are designated beneath each lane, and the positions of molecular weight markers are shown on the right.

imported into isolated chloroplasts, the expected 17-kD processed form is detected in both the thylakoid and stromal fractions, and seems to be localized preferentially in the stroma (Figure 3). Similarly, imported pSSU is detected primarily in the stroma, but some radioactivity also can be seen in the thylakoid fraction (Figure 3). Chloroplast envelope membranes do not sediment with the thylakoid membranes in this fractionation (Kohorn et al., 1986; Smeekens et al., 1986), and it is likely, therefore, that the thylakoid fraction is contaminated with stroma. Thylakoid preparations from pea chloroplasts were found to exhibit less association of the SSU with the thylakoid membranes, and so these were utilized for further import experiments, rather than those from *Lemna*, from which the pLHCP and pSSU genes used here were cloned (Kohorn et al., 1986; Tobin et al., 1984). Neither quantitative nor qualitative differences in import reactions have been observed between these chloroplast preparations, except that intact chloroplast yields are higher from peas.

Helix 3, but not 1 or 2, Can Cause Thylakoid Association

Lipophilic amino acid sequences by definition tend to associate with membrane fractions. The association of SSU3LHCP with the thylakoid could be mediated solely by the hydrophobic nature of the LHCP sequence, or by some other additional factor. A different sequence predicted to be of equal hydrophobicity to that of helix 3 (Eisenberg, 1984; Karlin-Neumann et al., 1985), but that was not required for stable thylakoid association (Kohorn et al.,

1986), was therefore fused to pSSU, and this fusion protein was assayed for its ability to associate with thylakoid membranes.

Helices 2 and 3 of LHCP are predicted to have equal hydrophobicities, whereas that of helix 1 is slightly less (Eisenberg, 1984; Karlin-Neumann et al., 1985). Nucleotide sequences, including these other two regions (helix 1, amino acids 94 to 140; helix 2, amino acids 150 to 190), were fused individually to the carboxy terminus of the pSSU coding region to create pSSU1LHCP and pSSU2LHCP, both of which are predicted to have an apparent molecular weight of about 24 kD. The abilities of all three of these fusion proteins and of LHCP and SSU to associate with thylakoid membranes are compared in Figure 4. Equal amounts of precursors were incubated with isolated thylakoid membranes under conditions that allow the correct insertion of pLHCP into the membrane, but do not provide processing activity (Cline, 1986). The direct incubation with thylakoids, an "insert" reaction, rather than with isolated chloroplasts, an "import" reaction, was used as a better assay for the effect of the LHCP sequences on thylakoid insertion because different fusion proteins can differ in their initial chloroplast envelope binding and import efficiencies (Kuntz et al., 1986; Wasmann et al., 1986; Keegstra and Bauerle, 1988; see also Figure 2). Figure 4 shows autoradiography of thylakoids that have been incubated with equal amounts of pSSU, pLHCP, pSSU1LHCP, pSSU2LHCP, and pSSU3LHCP. These membrane fractions were then washed with 2 M NaBr to remove proteins that were not tightly associated (Nechushtai and Nelson, 1984). Whereas pSSU and pSSSU1LHCP cannot be detected in these thylakoids, both pLHCP and pSSU3LHCP are seen as unprocessed forms. Strikingly, more pSSU3LHCP is detected than pLHCP. Only a very low level of pSSU2LHCP is seen to associate with the thylakoids.

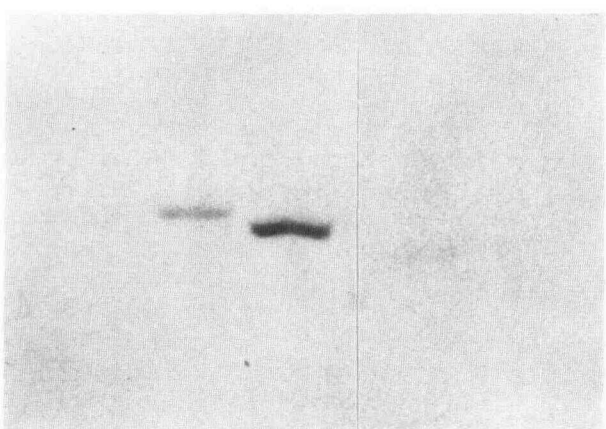

Figure 4. Incubation of Pea Thylakoids with SSU-LHCP Fusion Proteins.

Equal amounts of the fusion proteins pSSU1LHCP, pSSU2LHCP, pSSU3LHCP, pSSU, and pLHCP were incubated with isolated thylakoids and a stromal fraction in conditions that allow the insertion of pLHCP into the membrane. Thylakoids were washed with 10 volumes of 2 M NaBr and then analyzed by denaturing gel electrophoresis and fluorography. The names of the precursors incubated with thylakoids are shown beneath each lane.

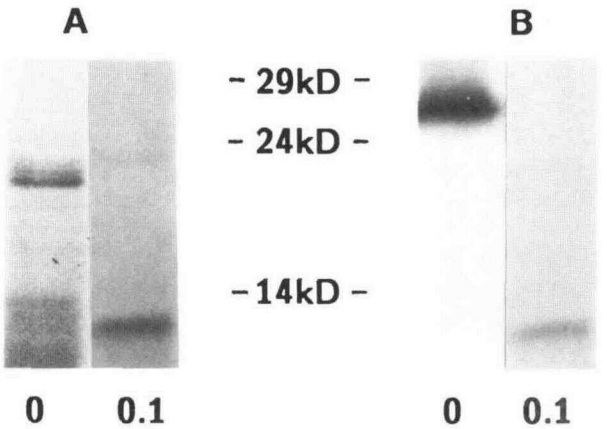

Figure 5. Trypsin Treatment of pSSU3LHCP and SSU3LHCP in Pea Thylakoid Membranes.

Thylakoid membranes containing SSU3LHCP from an import reaction **(panel A)** (see Figure 3) or pSSU3LHCP from an insert reaction **(panel B)** (see Figure 4), were incubated alone (lanes 0), or with 0.1 mg/ml trypsin (lanes 0.1) for 15 min and then analyzed by gel electrophoresis and fluorography. In panel A, lane 0.1 was exposed 3 times longer than lane 0. The positions of molecular weight markers are shown in the center.

Helix 3 Fusions Are not Inserted Fully into the Membrane

Treatment of isolated thylakoid membranes with trypsin can reveal whether an associated protein is protected by the lipid bilayer. Imported LHCP can be cleaved by 0.1 mg/ml trypsin such that only 2 kD are removed from the amino terminus, and this has been taken to indicate that, although the amino terminus extends into the stroma, much of LHCP is protected by its integration into the thylakoid membrane (Steinback et al., 1979; Figure 1). Figure 5 shows the trypsin sensitivity of the SSU3LHCP fusion in thylakoids. Thylakoids containing pSSU3LHCP (from an insert reaction, panel B) and SSU3LHCP (from an import reaction, panel A) were treated with trypsin, and a 12-kD labeled protein was found to accumulate in thylakoids from both reactions. Whereas the undigested pSSU3LHCP polypeptide can be immunoprecipitated by both anti-SSU and anti-LHCP antibodies, the 12-kD trypsin

product reacts only with the anti-SSU antibody (data not shown). The results of this experiment show that (p)SSU3LHCP is not inserted into the membrane in a way that allows protection of the LHCP fragment. Treatment of membranes containing (p)SSU3LHCP, or of pSSU3LHCP in a translation mix with carboxypeptidase A alone or even in the presence of detergent failed to produce any detectable protease digestion under conditions in which carboxypeptidase is active (data not shown).

A Mutant Lacking Amino-Proximal Amino Acids but Containing Helix 3 Inserts Incorrectly

A deletion mutation of pLHCP, D-pLHCP, that lacks 25 amino acids of a region that is thought to be located primarily in the lumen (amino acids 119 to 144, see Figure 1) is able to be taken up by isolated chloroplasts and insert into the thylakoid membrane, but it cannot assemble into LHCII (Kohorn et al., 1986). Intact thylakoid membranes containing imported, processed D-LHCP were subjected to protease treatments to determine the extent to which the protein was inserted into the lipid bilayer. Figure 6 shows the results of trypsin (lane B) and carboxypeptidase A (lane C) treatments. High concentrations (0.1 mg/ml) of trypsin remove about 4 kD from D-LHCP, consistent with the integral nature of the protein. Surprisingly, carboxypeptidase A removes about 10 kD, an amount that is predicted to include sequences spanning helix 3 and the carboxy terminus (amino acids 176 to 271, Figure 1). Thus, these regions of D-LHCP are apparently exposed in the stroma.

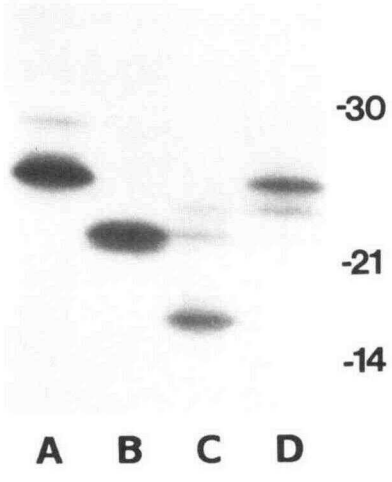

Figure 6. Import and Protease Digestions of a Deletion Mutant, D-LHCP.

A pLHCP deletion mutant, D-LHCP (Kohorn et al., 1986), that lacks amino acids 119 to 144 (Figure 1) was imported into isolated pea chloroplasts, and the thylakoids were treated with protease. Samples were then dissolved and subjected to electrophoresis in a denaturing 10% to 25% polyacrylamide gel, and fluorography. Lane A, no protease; lane B, 0.1 mg/ml trypsin; lane C, 0.1 mg/ml carboxypeptidase A; lane D, 0.1 mg/ml carboxypeptidase A and 1 mg/ml carboxypeptidase A inhibitor. Molecular weight markers ($\times 10^{-3}$) are shown on the right.

DISCUSSION

Models for LHCP in the Thylakoid Membrane

LHCP becomes an integral thylakoid membrane protein upon import into the chloroplast. Models for the topography of LHCP in the thylakoid predict either three (Karlin-Neumann et al., 1985; Bürgi et al., 1987) or four (Anderson and Goodchild, 1987) membrane-spanning α-helices. Although both models place the amino terminus in the stroma, the two views differ in two related points: (1) the second membrane-spanning helix of the four spanning segment model is placed entirely in the lumen by the other model and thus (2) the carboxy terminus of the four spanning segment model is stromal, rather than luminal, as predicted by the three spanning segment model. Helix 2 of the four spanning segment model is highly charged, and it is unclear how any secondary or tertiary structure could permit all of these residues to exist stably in a lipid environment. This region is predicted to be hydrophilic (Karlin-Neumann et al., 1985).

Treatment of thylakoids with carboxypeptidase A demonstrates that the carboxy terminus of LHCP is not accessible on the stromal side; detergent must be present in a concentration that solubilizes the thylakoid vesicles for this peptidase to digest LHCP. These data only prove that the carboxy terminus is buried on one side of the membrane, as detergent treatment may also unfold LHCP, thus providing access to the protease. Results of experiments involving the radioiodination of inside-out versus rightside-out vesicles agree with the LHCP orientation presented here (Bürgi et al., 1987). Contrary evidence suggesting that the carboxy terminus is stromal is based on agglutination studies that use high concentrations of antibody and could, therefore, be prone to misinterpretation (Anderson and Goodchild, 1987; McCrea et al., 1988). A picture of the true structure awaits the crystallization and x-ray diffraction analysis of LHCP (cf. Kuhlbrandt, 1984; Li, 1985). For the sake of discussion, the putative LHCP membrane-spanning regions will be named according to the model presented here.

Helix 3 Is Required for LHCP Stability

Deletion of a region within helix 3 renders LHCP capable of import, yet unstable within the chloroplast. This mutant protein (J-LHCP) can associate with the thylakoid, but it is

sensitive to protease digestion, and thus not fully integrated. These results indicate that helix 3 contains either a membrane-seeking sequence or a region that is essential for a required folding, important for the integration process. Since J-LHCP can still target to the thylakoid with reduced efficiency, remaining LHCP sequences must be able to dictate the sorting between the stroma and the membrane.

Helix 3 Causes Association with the Thylakoid

Analysis of fusion proteins indicate that the carboxy-terminal region containing helix 3 (amino acids 210 to 271), and not other LHCP sequences of similar predicted hydrophobicity, effects an association with the thylakoid membrane. Fusion of pSSU to LHCP residues 241 to 271, which comprise the carboxy tail of LHCP, allows the import of the fusion protein into the chloroplast, but the processed form is detected in both the stromal and thylakoid fractions. Thus, we infer that, although the region between amino acids 241 and 271 is important for correct insertion (Kohorn et al., 1986), there is a region between amino acids 210 and 240 that is important for specific association with the thylakoid.

More SSU3LHCP than LHCP itself associates with thylakoids, suggesting that the helix 3 segment of SSU3LHCP could be more free to interact with a membrane or effector molecules (Cline, 1986; Chitnis et al., 1987) than it is in the native protein, where it may be in a different or more buried conformation. Changes in the folding of helix 3 may be evidenced by its variable sensitivity to carboxypeptidase A in different protein contexts (compare LHCP, SSULHCP, and D-LHCP).

Additional Requirements for Integration

Trypsin digestion of SSU3LHCP in thylakoid fractions produces a 12-kD peptide containing SSU sequences. Therefore, helix 3 can only cause a peripheral association with the thylakoid, and alone cannot cause complete integration into the membrane. We do not know why the SSU sequence is protected from the protease, but we speculate that it might be the consequence of an association of SSU3LHCP with the large subunit of ribulose bisphosphate carboxylase. SSU3LHCP is, nevertheless, retained by thylakoids washed with 2 M NaBr, and is likely, therefore, to be associated tightly with the membrane.

Analysis of an additional deletion mutation of LHCP, D-LHCP, suggests that portions of LHCP can integrate into the thylakoid, whereas helix 3 remains in the stroma, accessible to carboxypeptidase. Thus, whereas helix 3 is required for LHCP integration, other LHCP sequences are required for the proper integration of helix 3. These results indicate that LHCP thylakoid targeting and integration require a complex interaction between a number of different protein domains. Helix 3 is required for correct LHCP insertion and can cause the normally soluble SSU to associate with the thylakoid membrane. Under other circumstances, helix 3 itself cannot integrate into the thylakoid, and requires the presence of additional regions within LHCP.

Recent work suggests that only some of the chloroplast targeting information resides in the transit peptide (Smeekens et al., 1986). A number of distinct signals within the transit peptide have been proposed to affect the sorting of mitochondrial proteins (Pfanner et al., 1988); yet it is increasingly evident that the ordered, energy-dependent refolding of a protein also plays an important role in this process (Verner and Schatz, 1988). Targeting to the thylakoid (versus the stroma) could be, in part, the consequence of hydrophobic segments preferring lipid environments. Perhaps refolding upon import allows the exposure of such segments. The removal of hydrophobic residues, as in J-LHCP, may result in a corresponding decrease in association with lipid. Indeed, LHCPs lacking either the first or second helix show reduced levels in the thylakoid membrane after import into isolated chloroplasts (Kohorn et al., 1986). Once at the thylakoid, necessary effector molecules (Cline, 1986; Chitnis et al., 1987) and pigments (Plumley and Schmidt, 1987) would allow further insertion.

The means by which a water-soluble precursor becomes an integral membrane protein may also require the involvement of pigments before integration can occur. Genetic studies imply that the presence of pigments in the chloroplast is required for the stability of LHCP (Bellemare et al., 1981). However, inclusion in the chlorophyll-protein complex LHCII is not a prerequisite for insertion into the thylakoid, as mutants that block LHCII assembly do not inhibit thylakoid insertion (Kohorn and Tobin, 1987). None of the mutants described here associates with LHCII, but it has not yet been possible to determine whether any pigments are bound to the protein monomers.

An analysis of the energy requirement for the LHCP integration and a detailed description of the folding pathways are now required. Additional factors involved in this process have been described recently (Cline, 1986; Chitnis et al., 1987), and these too must be included in the final mechanism.

METHODS

Protein Synthesis and Import

The in vitro expression and import of pLHCP into *Lemna* chloroplasts and the analysis of incorporated proteins by gel electrophoresis were as described (Kohorn et al., 1986). Import into isolated pea chloroplasts (Grossman et al., 1981), the insertion of precursors into isolated thylakoids (Cline, 1986), denaturing gel electrophoresis (Laemmli, 1970), and fluorography (Lasky and Mills, 1975) were also carried out by published procedures.

Fusion Protein Construction

A full-length cDNA encoding the precursor of the small subunit of ribulose, 1,5-bisphosphate carboxylase was constructed by combining a SalI-HindIII fragment of an incomplete *Lemna gibba* SSU cDNA that lacked the amino-terminal coding region, with a 160-bp BamHI-SalI fragment from a genomic clone SSU5 (Tobin et al., 1984). The BamHI-SalI-HindIII fusion was cloned into the BamHI-HindIII sites of pSP65, creating pSP65pSSU, and the plasmid was linearized with HindIII for expression of RNA and protein in vitro.

Fusion proteins were synthesized from derivatives of *L. gibba* pLHCP (Kohorn et al., 1986) and pSSU clones. The HindIII site of pSP65pSSU lies within the termination codon, and this site was eliminated by treatment with mung bean nuclease (Komalski et al., 1976) and the addition of a BamHI linker (CGGGATCCCG). This strategy allowed the addition of coding sequence to the carboxy terminus of pSSU at the new BamHI site. The BamHI linker contributes amino acids I and R to expressed proteins. Fragments of pLHCP containing 5′-BamHI termini created by Bal31 nuclease and linker addition (Kohorn et al., 1986) were added to the BamHI pSSU site to create coding regions for in-frame fusion proteins. LHCP sequences added were as follows (where amino acid [aa] 1 is the pLHCP initiating methionine): pSSU1LHCP, aa 94 to 271; pSSU2LHCP, aa 150 to 190; pSSU3LHCP, aa 210 to 271; pSSUCLHCP, aa 241 to 271. To create an in-frame coding sequence, the BamHI site of pSSU1LHCP was cleaved and treated with the Klenow fragment of DNA polymerase (Pharmacia LKB Biotechnology Inc.) and four dexoynucleotide triphosphates, and then ligated. pSSU1LHCP was linearized with BglII (Kohorn et al., 1986) for in vitro expression so as to truncate RNA and protein synthesis at position 140. pSSU2LHCP, pSSU3LHCP, and pSSUCLHCP were linearized with HindIII to provide in vitro templates for pSSU fusion proteins that contain LHCP amino acids 150 to 190, 210 to 271, and 241 to 271, respectively.

Protease Digestions

Isolated thylakoid membranes were adjusted to a chlorophyll concentration of 1 mg/ml with import buffer and treated with 0.1 mg/ml trypsin treated with tosylphenylalanyl chloromethyl ketone (Sigma) for 15 min, or 0.1 mg/ml PMSF-treated carboxypeptidase A (Sigma) and 1 mM PMSF for 60 min. When appropriate, carboxypeptidase A inhibitor (Sigma) or Triton X-100 (Sigma) were added to 1 mg/ml and 25 mg/ml, respectively, before addition of protease.

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