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Communication

Replacement of Histidines of Light Harvesting Chlorophyll *a/b* Binding Protein II Disrupts Chlorophyll-Protein Complex Assembly[1]

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ABSTRACT

Eukaryotic light harvesting proteins (LHCPs) bind pigments and assemble into complexes (LHCs) that channel light energy into photosynthetic reaction centers. The structures of several prokaryotic LHCPs are known and histidines are important for the binding of the associated pigments. It has been difficult to predict how the eukaryotic LHCPs associate with pigments as the structure of the major LHCP of photosystem II is not yet known. While each LHCPII binds approximately 13 chlorophylls the protein contains only three histidines, one in each putative transmembrane helix. Experiments that use isolated pea (*Pisum sativum* L.) chloroplasts and mutant LHCPII synthesized *in vitro* show that the substitution of either an alanine or an arginine for each histidine residue inhibits some aspect of LHCII assembly. The histidine of the first membrane helix, but not the second or third, may be involved in the transport across the chloroplast envelope. No histidine alone is essential for the insertion of LHCP into thylakoid membranes, yet arginine substitutions are more inhibitory than those of alanine. The histidine replacements have their most pronounced effect on the assembly of LHCP into LHCII.

The pigments that are found in photosynthetic membranes are present in highly ordered arrangements that allow the efficient capture of light energy (5, 18). Bchls[2] can be noncovalently bound to histidine residues within membrane spanning regions of polypeptides through the interaction of the Mg of the Bchl and the side group of the amino acid (18). Eukaryotic LHCPs show no primary amino acid sequence homology with their bacterial counterparts, and as no crystal structure is known for the former, it is difficult to predict how Chl is bound to LHCPs. The major LHCP of higher plants is a 28 kD protein that is thought to span the thylakoid membrane three times, bind approximately 13 Chls, and form LHCII (5). Examination of the proposed membrane spanning domains of LHCP shows that each approximately 20 amino acid segment contains or is proximal to one histidine residue, and these are the only histidines of the protein (7). In a number of plant species these histidines are either conserved or are replaced by asparagines (2) which have also been implicated in Bchl binding (18). Chl is required for the assembly and stability of LHCII in higher plants (5, 15), and it therefore is of interest to determine the role of histidines in LHCPII assembly. I show here that LHCPII histidines are important for the correct assembly of the Chl-protein complex LHCII in isolated pea chloroplasts. Histidine substitutions appear to be pleiotropic in effect and disturb the import across the chloroplast envelope, the integration of LHCPII into the thylakoid membrane, and also the assembly of LHCII.

METHODS

The *in vitro* expression and import of pea (*Pisum sativum* L.) pLHCP into pea chloroplasts and into isolated thylakoids, and the analysis of incorporated proteins by gel electrophoresis were as described (3, 6, 8, 11). The pea pLHCP coding sequence (compliments of W. F. Thompson) is contained within an *Eco*RI-*Hin*dIII restriction fragment cloned into pgem4 that also contains an origin of replication for M13 phage. Single stranded DNA was used for mutagenesis with a kit from Amersham Corp. and was resequenced after cloning. The histidines of helix 1, 2, and 3 that were replaced are at amino acid 65, 117, and 209, respectively, where amino acid 1 is the first residue of the mature protein.

RESULTS AND DISCUSSION

Radiolabeled precursor LHCP (pLHCP) can be synthesized from a cloned DNA template *in vitro*, imported into isolated chloroplasts, processed, and subsequently assembled into LHCII of the thylakoid membrane (6, 8). Mutations of pLHCP are readily characterized using this *in vitro* assay and a measure of their ability to import and assemble into LHCII can be determined. As some alterations do affect import itself, an additional assay that uses isolated thylakoids (3) can determine the direct effect of mutations on the insertion of LHCP into thylakoids and LHCII. Both assays were used to characterize histidine replacement mutants of pLHCP.

The histidine of each of the three membrane spanning regions 1, 2, and 3 (Fig. 1) was replaced by either an alanine, producing LHCP1A, 2A, and 3A, respectively, or an arginine, producing pLHCP1R, 2R, and 3R. Equal amounts of each of these altered radiolabeled pLHCPs were then imported into

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[2] Abbreviations: Bchl, bacterial Chl; LHCP, light harvesting, Chl *a/b* binding protein; LHC, light harvesting complex; LHCII, light harvesting complex of PSII.

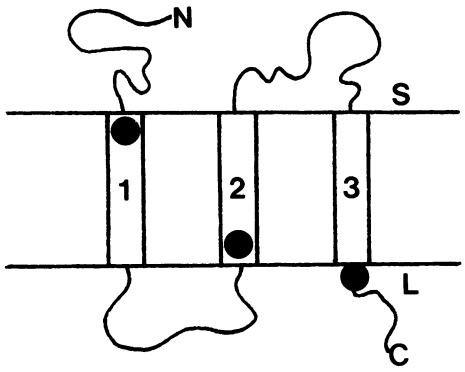

Figure 1. Predicted arrangement of LHCP in the thylakoid membrane (7). Boxes and numbers indicate membrane spanning sequences; horizontal lines denote stromal (S) and lumenal (L) membrane boundaries; (●), Position of histidine residues; N, amino terminus; C, carboxyl terminus.

intact chloroplasts (6, 8). The chloroplasts were subsequently reisolated, washed, and whole organelles were analyzed by denaturing gel electrophoresis (11) and autoradiography (12). The results are shown in Figure 2. LHCP is detected as a processed 28 kD protein within the chloroplast (6, 8) (lane +) but only LHCP2A and LHCP3A (lanes 2A and 3A) accumulate to a level similar to that of the wild type. LHCP1A, 1R, 2R, and 3R are only detected at very reduced levels. The 32 kD pLHCP and the mutant pLHCPs are also found in each fraction (Fig. 2). These represent precursor bound to the chloroplast outer envelope as they can be digested by external protease treatment of intact organelles (4; data not shown). It is evident that different amounts of precursor are bound to the outer envelope, suggesting that the mutations affect the binding and efficiency of import.

One mutant, pLHCP1A, that did not accumulate as a processed protein, was analyzed at shorter and longer times of import to determine whether it was impaired in import ability or if it was rapidly degraded once within the chloroplast. Import reactions for both pLHCP and pLHCP1A were terminated at 0, 5, 10, 20, or 40 min of incubation by the addition of nigericin (4) and intact chloroplasts were reisolated, washed, denatured, and subjected to electrophoresis and autoradiography. The results are shown in Figure 3. pLHCP binds to the organelle and then appears as a processed band within 5 min of incubation. pLHCP1A, however, accumulates at a slower rate on the outside of the chloroplast (as it is sensitive to external protease treatment, data not shown) and only very low levels of processed 28 kD LHCP1A are detected. pLHCP deletion mutants (8) and chimeric proteins (17) can have altered binding and import efficiencies, but experiments described here show that a single amino acid change within the mature peptide can also be inhibitory, perhaps stressing the importance of tertiary structure in the initial events of correct chloroplast targeting. It remained possible that LHCP1A was also rapidly degraded once within the chloroplast, and because of the additional block in import, a more direct assay for the integration into thylakoids and LHCII was needed to characterize the mutants.

pLHCP will insert into isolated thylakoids in the presence of a stromal extract and ATP (3) and this 'integration' assay bypasses the import step across the chloroplast envelope. Following the incubation with pLHCP and the mutant pLHCPs, the thylakoids were treated with trypsin to remove nonintegral membrane proteins. When LHCP is correctly inserted in the thylakoid only a 2 kD (16) and to a lesser extent a 4 kD (3, 8) region are cleaved from the amino-terminus, producing 26 and 24 kD proteins, respectively. The protease treated thylakoids from an integration assay using pLHCP and each of the histidine mutations are shown in Figure 4A. pLHCP is correctly inserted into the thylakoid since a 26 and 24 kD band remain after trypsin treatment (lane +, dLHCP1, and 2, respectively). Histidine replacement mutants are also protected by the membrane from the protease treatment but the levels of integration are lower than that of wild type (lanes 1A–3R). pLHCP2R and 3R accumulate the least. Two double mutants that replace the histidine of both helices 2 and 3 with alanine (LHCP23A) or arginine (LHCP23R) were also assayed and these can integrate, but again at levels lower than that of pLHCP. The reduced levels of accumulation by the mutants is not the result of increased turnover within the chloroplast extract as equal levels of radioactive pLHCPs are detected in thylakoids that have not been treated with trypsin (data not shown). Thus, the levels shown in Figure 4A represent relative amounts of membrane insertion by each of the mutants.

LHCII was purified (8, 14) from the trypsin-treated thylakoid membranes that contained inserted LHCP and mutant LHCP (shown in Fig. 4A). Trypsin-trated rather than native thylakoids were fractionated to ensure that any detected protein was indeed inserted and not sticking to the complex. The LHCIIs were then subjected to denaturing gel electrophoresis and autoradiography, and the results are shown in Figure 4B. While pLHCP is readily assembled into LHCII (lane +), pLHCP2A and 3A are only detected at reduced levels, and the remaining mutants, including pLHCP23A, are not assembled. (Only the 24 kD degradation product, dLHCP2 in Fig. 4B, is detected as trypsin digestion continues for longer times and in the presence of mild detergent during the isolation of LHCII.) The presence of the histidine in helix 1 is therefore essential for LHCP to assemble into LHCII, and neither an alanine nor an arganine can substitute. Low levels of LHCP2A and 3A may be able to assemble into LHCII (lanes 2A and

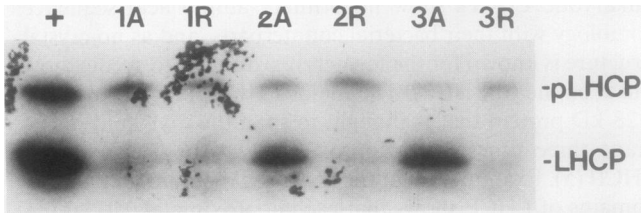

Figure 2. Import of pLHCP and histidine substitution mutants into isolated, intact chloroplasts. Shown is an autoradiograph of a denaturing gel (11) containing chloroplasts incubated with a mutant pLHCP that is indicated above each lane. Equal amounts of each pLHCP were added to equal numbers of intact pea chloroplasts (Chl equivalent) and incubated for 10 min at 25°C in the light in import buffer (6). Reactions were terminated by a centrifugation for 3 min through 35% Percoll, a rinse in import buffer, and then denaturation.

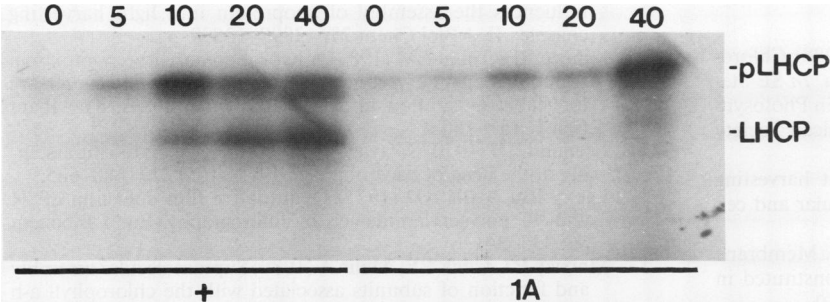

Figure 3. Time course of import of pLHCP and pLHCP1A into isolated chloroplasts. Equal aliquots were removed from an import reaction containing either pLHCP (+) or pLHCP1A (1A) at 0, 5, 10, 20, and 40 min, washed as in Figure 2, and subjected to denaturing gel electrophoresis and autoradiography.

3A) as they still each contain the histidine of helix 3 or 2, respectively. Removal of both histidines (LHCP23A) causes a complete block in assembly (lane 23A). Thus, the histidines of helices 2 or 3 alone are not essential, but their combined absence inhibits LHCII assembly.

Replacement of histidine 2 or 3 with arginine is also completely inhibitory (lane 2R, 3R), indicating that the presence of an additional charge in the membrane is disruptive. Indeed, a specific charge distribution between the three proposed helices is essential for LHCII assembly, but not for integration into thylakoids (9). The presence of a new arginine could also lead to steric hindrance during LHCII assembly.

Histidine residues in each of the three proposed membrane spanning regions of LHCP thus appear to be required for the proper assembly of LHCII. Neutral alanine substitutions are more permissive than those of charged arginines, possibly due to the requirement of a balanced charge distribution within LHCP (9). Many studies show that LHCP will not form stable LHCII in chloroplasts that lack certain pigments (5) yet it has not been possible to develop an assay to determine whether individual LHCPs bind Chl (14). The results described here are only consistent with the idea that histidines bind Chl, and histidine substitutions are equally likely to disrupt a structure that forms independently of pigments. There is, however, further correlative evidence that suggests that histidines bind Chl; the amino acid sequences of several LHCPs (2) predict membrane-spanning regions that lack the required histidine, but instead, contain asparagine, and asparagines are also thought to bind Bchls on the chlorosomes of *Chloroflexus aurantiacus* (18). It is likely that the three histidines of LHCPII alone would not be sufficient to bind all of the Chls as there are approximately 13 Chls per LHCPII (5, 13). Bchls of chlorosomes can be present as large oligomers that are maintained by pigment-pigment interactions (1) and this arrangement coupled with protein-Chl binding could account for the observed stoichiometries of higher plant LHCPs and Chls.

LHCP can integrate into the thylakoid independently of LHCII assembly (3, 8) and it has not been determined whether pigments are required for the integration process itself. Indeed, the means by which the water soluble LHCP becomes lipid soluble is not understood (10). Mutants that lack histidines insert into thylakoids only at reduced efficiencies and this may be a reflection of either an alteration of required protein structure, or perhaps a role of Chl in the solubility transition of LHCP.

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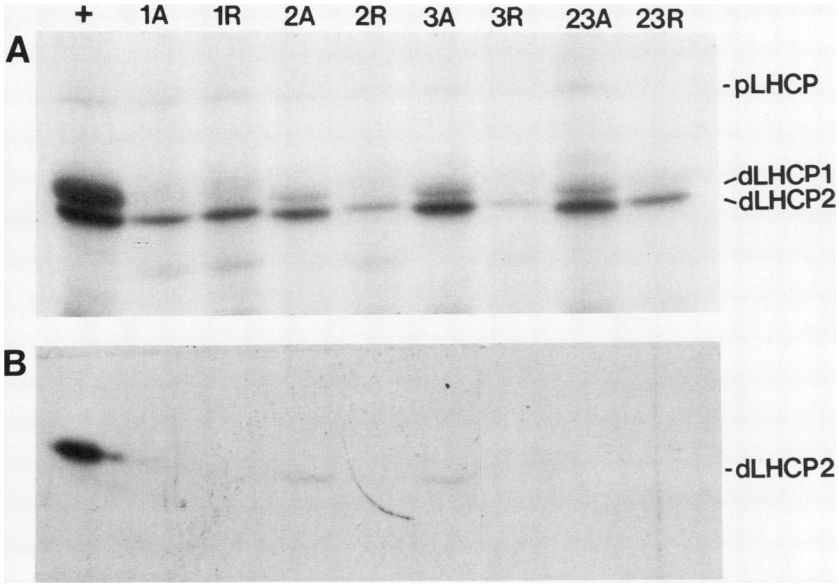

Figure 4. Insertion of pLHCP and pLHCP mutants into isolated thylakoids (A) and LHCII (B). Insertion reactions contained chloroplast lysate (3), ATP and one of the labeled mutant pLHCPs (1A–23R) or wild type (+) protein, as indicated above each lane of the autoradiograph. Reactions were for 30 min at 25°C. Thylakoids were washed and treated with 0.1 mg/mL of trypsin for 15 min at 25°C and then immediately denatured (panel A) or fractionated to yield LHCII (8, 14) and then denatured (panel B). dLHCP1, dLHCP2; 26 and 24 kD trypsin degradation products, respectively.

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