Integration of a Chlorophyll-binding Protein into Escherichia coli Membranes in the Absence of Chlorophyll*

(Received for publication, December 21, 1990)

Bruce D. Kohorn‡ and Andrea H. Auchincloss
From the Department of Botany, Duke University, Durham, North Carolina 27706

The mechanism by which a protein integrates post-translationally into a membrane can involve the composition of the membrane itself, domains within the inserting polypeptide, and a number of associating proteins. Some integral membrane proteins do not accumulate to normal levels when certain pigments are deficient, and this has been interpreted to mean that such proteins may be rapidly degraded when not in a correct complex. Alternatively, pigments could facilitate the movement of some proteins from an aqueous to a lipid environment. To determine whether chlorophyll is absolutely required for the membrane integration of the light-harvesting chlorophyll-binding protein (LHCP) of chloroplast thylakoid membranes, we have expressed LHCP in Escherichia coli that lacks photosynthetic pigments. LHCP is targeted to the bacterial inner membrane by the addition of a bacterial signal peptide and cannot be extracted from these membranes by NaOH, NaBr, or Na2HCO3 but is extracted by 0.2% Triton X-100. Treatment of isolated right-side-out and inside-out bacterial inner membrane vesicles with trypsin reveals that only the amino terminus of LHCP is exposed on the cytoplasmic face, and the remaining portion of the protein is inaccessible. Treatment of the inside-out vesicles with trypsin followed by alkaline extraction shows that LHCP is intrinsic to the membrane and is not anchored solely by the bacterial signal peptide. Chlorophyll, therefore, is not required for LHCP to integrate into a membrane, but in the absence of these pigments this process is observed to be inefficient.

Integral membrane proteins can be inserted into lipid bilayers through either co- or post-translational processes (1-3). Eukaryotic proteins synthesized on the endoplasmic reticulum utilize a signal recognition particle that arrests translation, binds specific receptors on the endoplasmic reticulum, and allows the threading of a nascent polypeptide into the membrane (2). More recently a number of chaperonin-like factors have been identified that can mediate a post-translational insertion of a protein into the endoplasmic reticulum (2). Bacterial inner membrane proteins also can interact with a signal recognition particle (4) or a number of distinct peptide-binding chaperonins (3) that mediate the insertion of a protein into the membrane. In these prokaryotic systems as well, insertion is not necessarily co-translational. Indeed, the M13 phage coat protein can insert spontaneously in the absence of other factors (5). It appears, therefore, that there is a wide spectrum in the complexity of events that describe the insertion of membrane proteins. Within this spectrum are pigment-binding membrane proteins that, in some manner, require the addition of pigments for their accumulation in the membrane (6, 7). It is unclear, however, what role these pigments have in the insertion of proteins into membranes.

The insertion of the integral membrane light-harvesting chlorophyll a/b-binding protein (LHCP) into the thylakoid membrane of the chloroplast is clearly post-translational as it is synthesized in the cytoplasm (6). Upon entering the chloroplast, LHCP is bound by a stromal factor that appears to be essential for the eventual insertion of LHCP into the thylakoid (8). Sequences within a carboxyl-terminal hydrophobic region contain thylakoid targeting information but alone are not sufficient for membrane integration (9). The events that bring about the post-translational integration of LHCP are undefined, but it is clear from many studies that chlorophyll and other photosynthetic pigments are required for the accumulation of a stable, integrated LHCP (6, 10, 11). Cells of higher plants that are deficient in chlorophyll b have greatly reduced levels of LHCP, even though the protein is likely to be synthesized (12). These observations have led to the widely accepted idea that chlorophyll b is required for the stability of LHCP and that protein not correctly complexed with pigment is rapidly degraded. An additional interpretation, however, is that chlorophyll facilitates the integration of LHCP into the lipid bilayer and that only the nonintegrated protein is specifically degraded. Indeed, the addition of chlorophyll to a membrane protein could aid in the transition of the protein from an aqueous to a lipid environment (13).

The in vitro import of small amounts of LHCP into chloroplasts isolated from a chlorophyll b-deficient plant has shown that LHCP integrates into these thylakoid membranes (14). However, this study does not eliminate the possibility that small amounts of chlorophyll remain to allow insertion or that the insertion is inefficient. To determine whether chlorophyll is absolutely required for the insertion of LHCP into a membrane, LHCP has been expressed in Escherichia coli. Some of the LHCP that is targeted to the inner membrane with a bacterial signal peptide becomes an integral membrane protein in this bacterium. Chlorophyll is not, therefore, essential for LHCP to become embedded in a lipid bilayer.

EXPERIMENTAL PROCEDURES

LHCP Gene Constructs—A DNA fragment encoding a Lemna LHCP (15) was synthesized using the polymerase chain reaction and

* This work was supported by National Institutes of Health Grant GM39696-01 and by a Pew scholar award (to B. D. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom reprint requests should be addressed: Dept. of Botany, Duke University, Durham, NC 27706. Tel.: 919-684-6141; Fax: 919-684-5412.

The abbreviations used are: LHCP, light-harvesting chlorophyll a/b-binding protein; sp, signal peptide; R50, right-side-out; ISO, inside-out; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
two oligonucleotides (5'-AGAGCTTCCATGCAAGGGAGAGCCG-3' and 5'-AGACGTGGGGCTGATTCACCTTAGGGGCGG-3') that amplified only the mature LHCP-coding sequence (amino acids 36-271), added an alanine codon after the methionine 36 codon, and created NcoI restriction enzyme sites at either end, and the amplified NcoI-digested fragment was cloned in the correct orientation into the NcoI site of pET-8C/LHCP, upstream of the LHCP-polymerase under control of IPTG (16). Expression was induced by the addition of IPTG to the bacterial culture. One liter of IPTG-induced cells was harvested and washed in 40 ml of 0.2 M NaPO₄, pH 7.5, and sonicated on ice with a Branson microtip at setting 4, with three 15-s pulses every 60 s. Care was taken not to heat the sample. The sonicate was centrifuged at 5,000 g for 10 min, producing a pellet (P1) and a supernatant. P1 was resuspended in 10 mM NaPO₄, pH 7.5, to 0.1 volume of the supernatant's volume. The supernatant was centrifuged again at 20,000 × g for an additional 10 min to clear remaining cell debris and protein bodies (19). The resulting supernatant (S1) was then centrifuged at 100,000 × g for 1 h producing a membrane pellet (P2) that was resuspended in 0.1 volume of the supernatant's volume in 10 mM NaPO₄, pH 7.5, and analyzed immediately. Samples loaded on gels contained equivalent fractions of total cell lysates. Trypsin was added to a final concentration, as indicated, for 10 min at 25 °C. Soybean trypsin inhibitor was added to 1 mg/ml after trypsin treatments. 0.1 N NaOH, 2 mM NaPO₄, pH 7.5, and sonicated bacterial cells were fractionated by centrifugation at 5,000 g for an additional minute, yielding an S1 pellet (S1) but not in the supernatant (TS). Thus, LHCP is not extracted by high concentrations of detergent and is likely confined to inclusion bodies, a finding similar to that for the precursor of LHCP (10, 27).

Expression of Signal Peptide LHCP—Targeting of proteins to the E. coli membrane is facilitated by an amino-terminal signal sequence of 15-25 amino acids fused to the mature protein (17). For example, the signal peptide from the maltose-binding protein (17) was fused to the amino terminus of mature LHCP (see "Experimental Procedures"). We then determined whether targeted LHCP could become an integral membrane protein. Proteins that were fractionated from E. coli expressing the 3-kDa sp-

\[ \text{LHCP} \rightarrow \text{sp-LHCP} \]

Expression of LHCP—The coding region for the mature 28-kDa LHCP was cloned into a bacterial expression vector and introduced into E. coli. After inducing the expression of LHCP, whole cell proteins prepared by sonication were fractionated by denaturing gel electrophoresis (22), transferred to nitrocellulose (26), and LHCP was detected using polyclonal LHCP antisera. Fig. 1. LHCP, shows the results of the cell fractionation and immunodetection. The pelleted LHCP was correctly expressed as the 28-kDa protein detected in a sonicated whole cell extract (lane W) co-migrated with LHCP of isolated thylakoid membranes (Fig. 1, lane TR). The sonicated bacterial cells were fractionated by centrifugation at 5,000 × g such that membranes and cytoplasm remain in the supernatant and cell walls, unbroken cells, and inclusion bodies are pelleted (19). LHCP is detected only in the pellet of this low speed centrifugation (P1) but not in the supernatant (S1). The pellet was resuspended, Triton X-100 was added to 1% for 10 min at 25 °C, and after a 5,000 × g centrifugation, LHCP was detected in the pellet (TP) but not in the supernatant (TS). Thus, LHCP is not extracted by high concentrations of detergent and is likely confined to inclusion bodies, a finding similar to that for the precursor of LHCP (10, 27).

Production of Carboxyl-terminal LHCP Antibodies—The peptide NH₄-CSYATNFVPGK-COOH that represents the carboxyl-terminal amino acids of a pea LHCP II (23) plus an additional cysteine at the amino terminus of the peptide was synthesized by David Klapner (University of North Carolina, Chapel Hill) on a Biosearch model 9500 peptide synthesizer according to Merrifield (24) and the manufacturer's instructions. The eight carboxyl-terminal amino acids are conserved between Lema and pea. The peptide (13) was coupled to Keyhole Limpet Hemocyanin (Sigma) using succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Pierce Chemical Co.) and autoradiography as described (25). Polyclonal antisera to gel-purified LHCP and to E. coli Sec Y were the generous gifts of K. Cline and W. Wickner, respectively.

Results
brane protein (29, 30). sp-LHCP appears not to be processed by bacterial signal peptidase as inclusion bodies was treated with different amounts of Triton X-100 and then centrifuged. The supernatant was centrifuged again at 100,000 g to clear residual cell debris and inclusion bodies to produce an S1 fraction (19). Ninety percent of the 31-kDa protein pellets at 5,000 x g (lane P1), but unlike LHCP, 10% is also detected in a 20,000 x g supernatant (lane S1). Thus sp-LHCP fractionates predominantly with inclusion bodies and unlysed cells, but a portion remains with a cytoplasmic membrane fraction. The 20,000 x g supernatant (S1) was centrifuged again at 100,000 x g to pellet the membranes, and the supernatant (S2) and pellet (P2) are also shown in Fig. 1, spLHCP. sp-LHCP is detected only in the pelleting material. The 100,000 x g P2 pellet was resuspended and treated with either 0.1 N NaOH or with 0.25% Triton X-100 (29, 30). The two samples were then centrifuged again at 100,000 x g, and the pellet and supernatant were analyzed by denaturing gel electrophoresis. sp-LHCP is detected in NaOH-treated membranes (lanes NP versus NS) and is found in the supernatant of detergent-treated membranes (lanes TS versus TP). Thus, sp-LHCP has the characteristics of an integral membrane protein (29, 30). sp-LHCP appears not to be processed to the expected 29-kDa size by bacterial signal peptidase as no mature LHCP is detected in any fraction. Proteins less than 31 kDa in molecular mass are seen (e.g. Fig. 1, spLHCP, lanes W, S1, and P1), but these may represent products of general proteolysis.

Detergent Solubilization of Membranes—The ability of detergent to solubilize sp-LHCP from the membrane fraction was investigated in more detail. The membrane fraction (S1) was treated with different amounts of Triton X-100 and then centrifuged at 100,000 x g. Fig. 2, M, shows the analysis of the resulting pellets and supernatants. sp-LHCP is partly extracted from the membranes by 0.2% Triton X-100 and fully extracted by 0.5%. The LHCP of thylakoid membranes shows a similar solubilization pattern (not shown). To eliminate the possibility that the LHCP detected in the membrane fraction was due to contaminating inclusion bodies or unbroken cells, we determined whether the pellet of the first 5,000 x g spin (Fig. 1, spLHCP, lane P1) could be solubilized with Triton X-100. Fig. 2, I, shows the results of the detergent treatments followed by a more gentle 10,000 x g spin to produce a pellet (P) and a supernatant (S). No detergent concentrations solubilize this fraction as only the pellets contain sp-LHCP. Since the inclusion bodies pellet after Triton X-100 extraction and a low speed centrifugation, they cannot account for the detergent-soluble sp-LHCP detected in the membrane fraction.

Protease Treatment of Membranes—Treatment of a membrane with trypsin can reveal whether an associated protein is protected by the lipid bilayer. Trypsin treatment of thylakoid membranes cleaves only 2 kDa from the amino terminus of LHCP (31, 32), and this is taken to indicate the integral nature of the protein. E. coli membranes containing sp-LHCP were also treated with trypsin to determine the extent to which LHCP would be protected from proteolysis. The results of proteolysis of both thylakoids and bacterial membranes and the subsequent detection of LHCP with polyclonal antisera are shown in Fig. 3. LHCP in thylakoids (lane 1) is reduced by 2 kDa in size by 0.1 mg/ml trypsin (lane 2) but is completely digested in the presence of 0.5% Triton X-100 and 0.1 mg/ml trypsin (lane 3). Treatment of the bacterial membranes with 0.001, 0.01, or 0.1 mg/ml trypsin (Fig. 3, lanes 4–7, respectively) results in the production of an ~26-kDa LHCP. The 26-kDa protein comigrates with the trypsin-treated LHCP of thylakoid membranes (lane 2), and the size is consistent with a trypsin cleavage that removes the bacterial sp and the amino terminus of LHCP. Complete proteolysis of sp-LHCP is seen in the presence of 0.5% Triton X-100 and 0.1 mg/ml trypsin (lane 8). Uncleaved sp-LHCP is also detected in each lane as this sonicated membrane fraction includes inside-out, right-side-out, and mosaic vesicles (19, 33) (see below).

LHCP Carboxyl Terminus Is Protected from Protease—To determine which portion of the sp-LHCP was removed by trypsin, antiseraum raised to the 10 carboxyl-terminal amino acids of LHCP was used to detect the proteolysis fragments shown in Fig. 3, lanes 5–7. This antiseraum detects both a 28-kDa protein in thylakoid membranes (Fig. 3, lane 9) and a trypsin-cleaved thylakoid LHCP that lacks the amino terminus (Fig. 3, lane 10). The carboxyl-terminal antiseraum is specific to the last 10 amino acids of LHCP. The carboxyl-terminal antibody also detects the 26-kDa protein that accumulates after the bacterial membranes are digested with trypsin (Fig. 3, lanes 11–14). The 26-kDa protein must, therefore, contain the LHCP carboxyl terminus, and by default the amino terminus is the region that is proteolysed. An ~21-kDa protein is also detected by both the polyclonal and the carboxyl-terminal antisera in proteolyzed bacterial membranes (Fig. 3, lanes 6, 7, 13 and 14), and this corresponds in size to a cleavage ~7 kDa from the mature LHCP amino terminus. There is indeed a predicted trypsin cleavage site in this location (23), and upon extensive proteolysis of LHCP in thylakoids (Fig. 3, lane 2) (8, 9) this cleavage product can also be detected.

![Fig. 2. Triton X-100 extraction of membranes (M) and inclusion bodies (I). LHCP was detected as in Fig. 1 in protein fractions that were treated before a 100,000 x g (M) or 10,000 x g (I) centrifugation with the amounts of Triton X-100 indicated beneath the figure. P and S refer to the pellet and supernatant after centrifugation.](image-url)

![Fig. 3. The amino terminus of sp-LHCP is proteolyzed by trypsin. Lanes 1–8 detection of LHCP with polyclonal antisera; lanes 9–14, with antiserum to LHCP carboxyl terminus. Isolated thylakoids were not treated (lanes 1 and 9) or treated with 0.1 mg/ml trypsin (lanes 2 and 10) or trypsin and 0.5% Triton X-100 (lane 3). The bacterial membrane fraction was treated with 0 (lanes 4 and 11), 0.001 (lanes 5 and 12), 0.01 (lanes 6 and 13), 0.1 mg/ml trypsin (lanes 7 and 14), or 0.1 mg/ml trypsin and 0.5% Triton X-100 (lane 8). Lines on the right of the figure indicate M, x 1,006 from top to bottom, 30 and 21.5, respectively.](image-url)

1 A. H. Auchincloss, unpublished observations.
While there are several topologies predicted for LHCP in the thylakoid membrane (15, 34, 35) it is only clear that the amino terminus is exposed to the stroma (31, 32). To determine on which surface of the bacterial membrane the amino terminus of LHCP is exposed, RSO and ISO bacterial inner membrane vesicles were prepared. These membrane vesicle preparations were treated with trypsin or washed with NaOH, NaBr, or Na₂HCO₃ and then subjected to a high speed centrifugation to pellet the membranes. Both the high speed pellet and supernatant were denatured and analyzed in a Western blot using polyclonal LHCP antiserum. Antiserum to the amino terminus of the integral inner membrane protein Sec Y (36, 37) was also used on these samples to verify the orientation and intactness of the membrane vesicles. The results of this analysis are shown in Fig. 4. In sodium dodecyl sulfate-polyacrylamide denaturing gels, Sec Y migrates as a 44-kDa protein (Fig. 4, lane 1). In RSO vesicles, Sec Y is not cleaved by trypsin (lane 3) and pellets with the membranes that have been treated with protease, NaOH, NaBr, or Na₂HCO₃ (lanes 3–7, respectively). In ISO vesicles, Sec Y can be cleaved by trypsin to yield a 28-kDa degradation product (lane 3), which still pellets with the membranes (lanes 3 versus 4). This is in agreement with previous reports that only a portion of Sec Y is exposed on the cytoplasmic face of the membrane (36, 37). A fraction of Sec Y is resistant to proteolysis in ISO vesicles (lane 3), indicating that the preparation may be contaminated with other membranes of alternate orientation. Sec Y still pellets with the membranes after the ISO vesicles have been washed with NaOH, NaBr, or Na₂HCO₃ (lanes 5–7, respectively), and this is also characteristic of an integral membrane protein. Analysis of the same membrane preparations with LHCP antiserum show that LHCP and Sec Y have similar characteristics. LHCP is resistant to proteolysis in RSO vesicles but is cleaved by trypsin into a 26-kDa protein in ISO vesicles (lanes 3). Like Sec Y, some LHCP is resistant to trypsin. Both the intact and 26-kDa proteolyzed LHCP pellet with the membranes in RSO and ISO preparations even after trypsin, NaOH, NaBr, or Na₂HCO₃ treatments (lanes 3 and 5–7, respectively), indicating that LHCP is an integral membrane protein. LHCP is also detected in the supernatant of pellet, untreated ISO membranes (lane 2) and therefore also in trypsin-treated ISO membranes (lane 4). The release of sp-LHCP from the ISO preparation into the supernatant may be due to a disruption of the ISOs during resuspension because the initial membrane fractionation from the sucrose gradient and high speed pellet (see “Experimental Procedures”) contained all of the sp-LHCP not in inclusion bodies (Fig. 3). Indeed even the extremely hydrophobic Sec Y protein can be detected in these supernatants upon longer exposures of the Westerns (Fig. 4, lanes 2 and 4). The observed degradation of LHCP in ISO fractions varies with experiments and is likely due to a general proteolysis during the extensive purification steps but cannot be reduced by proteinase inhibitors. Nevertheless, these results indicate that sp-LHCP can integrate into the bacterial inner membrane such that the amino terminus is exposed on the cytoplasmic face.

Trypsin treatment of the inside-out vesicles removes the signal peptide from LHCP, and the degradation product remains with the membrane fraction after centrifugation. We next determined whether this trypsin-treated LHCP is integral to the membrane rather than peripherally associated. We therefore treated ISO membrane vesicles with trypsin to remove the bacterial signal peptide and then washed with NaOH to determine whether LHCP could be extracted from the membrane fraction. The 100,000 × g pellet and supernatant of this treatment are shown in Fig. 5. Both the native sp-LHCP (lane a) and the trypsin degradation product of LHCP (lane c) remain in the pellet after alkaline extraction, and this demonstrates that the LHCP sequences themselves are integral to the membrane and that the bacterial signal peptide is not serving as the sole membrane anchor.

**DISCUSSION**

sp-LHCP can associate with the bacterial inner membrane such that the protein is resistant to extraction with NaOH, NaBr, and Na₂HCO₃ but is extracted by 0.2% Triton X-100. The amino terminus of LHCP is only accessible to added protease on the cytoplasmic face of the inner membrane. Proteolysis of sp-LHCP produces a 26-kDa membrane protein that according to its size must necessarily lack the aminoterminal bacterial signal peptide. This 26-kDa protein is not extracted from ISO membrane vesicles by alkaline treatment, and LHCP sequences themselves must therefore be integral to the membrane. As at least a portion of the bacterial signal peptide is likely to be on the cytoplasmic face, this may explain why sp-LHCP is not processed by the periplasmic signal peptidase (1).

Bacterial signal peptides direct proteins to the E. coli inner membrane, but the nature of the mature portion of the polypeptide is an essential determinant in whether the protein will become integral to or be translocated through the membrane (1, 3). The results described here demonstrate that the mature region of LHCP does not require chlorophylls to integrate into a membrane.

There may be several reasons why a bacterial signal peptide...
Light-harvesting Protein Integration into Membrane

is required for LHCP to accumulate as an integral membrane protein. The simplest of these is the need to target LHCP to the membrane where it can integrate either spontaneously or with the aid of the translocation machinery. Alternatively, the bacterial signal peptide may be able to substitute partially for a chloroplast stromal factor that is required in vitro for the integration of LHCP into thylakoid membranes (8). Interestingly, proteins targeted to the lumen of the thylakoid contain bacterial-like signal peptides (40) and do not require stromal factors for translocation across the thylakoid (41). It will be important to determine whether the addition of a signal peptide to LHCP can obviate the need for the stromal factor in the insertion of LHCP into thylakoid membranes and whether there are indeed more similarities between the bacterial and chloroplast machineries than have been previously suggested (40, 42).

Although only a small fraction of sp-LHCP associates with the membranes, chlorophyll may nevertheless play a role in protein translocation. Perhaps the insertion of LHCP into thylakoids could be likened to an equilibrium reaction that is partly driven in one direction by the stabilization of the end product by chlorophyll. The absence of chlorophyll may shift the equilibrium and result in an insufficient integration process. In plants that have insufficient chlorophyll, newly imported stromal LHCP may be degraded (6), while in E. coli the cytoplasmic sp-LHCP forms protein bodies. The existence of membrane-bound LHCP in Chlamydomonas strains lacking chlorophyll b (38) may reflect the lack of a stromal degradation mechanism in this alga and strengthens the concept that LHCP integration does not require chlorophylls. Also consistent with these results presented here is the observation that histidines of LHCP, putative chlorophyll ligands, are not essential for the insertion of LHCP into isolated thylakoid membranes (39). The observation that only ~10% of LHCP becomes an integral membrane protein in E. coli could also be due to the abundance of over-expressed LHCP; perhaps the bacterial transport system is limited in its capacity. This latter explanation seems less likely as the membrane LHCP is less abundant than other membrane proteins such as Sec Y.

The in vitro reconstitution of light-harvesting complexes from purified protein and pigments requires lipid or high concentrations of detergents (10, 11). It is therefore unlikely that a light-harvesting complex can form before LHCP becomes membrane-associated, but the possibility remains that chlorophyll or chlorophyll precursors could attach to LHCP before LHCP integrates into the thylakoid membrane. Indeed this may occur for chloroplast-encoded proteins that are synthesized on thylakoid membrane-associated ribosomes (43). Future experiments with reconstituted bacterial transport systems and purified pigments may be instructive in determining the full role of chlorophylls in the insertion of LHCP into a membrane.

Acknowledgments—We would like to thank Tracy Smith for stimulating discussions, Audrey Alexander for invaluable help, Ken Cline and Bill Wickner for antibodies, Bob Webster for the maltose-binding protein gene, and Jim Siedow, Cyril Kaspi, and Nick Gillham for advice.

REFERENCES