The Chlorophyll a/b-binding Protein Inserts into the Thylakoids Independent of Its Cognate Transit Peptide*

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In order to determine if the cognate transit peptide of the light-harvesting chlorophyll a/b-binding protein (LHCP) is essential for LHCP import into the chloroplast and proper localization to the thylakoids, it was replaced with the transit peptide of the small subunit (S) of ribulose-1,5-bisphosphate carboxylase/oxygenase, a stromal protein. Wheat LHCP and S genes were fused to make a chimeric gene coding for the hybrid precursor, which was synthesized in vitro and incubated with purified pea chloroplasts. My results show that LHCP is translocated into chloroplasts by the S transit peptide. The hybrid precursor was processed, and most importantly, mature LHCP did not remain in the stroma, but was inserted into thylakoid membranes, where it normally functions. Density gradient centrifugation showed no LHCP in the envelope fraction. Hence, the transit peptide of LHCP is not required for intraorganellar routing, and LHCP itself contains an internal signal for localization to the correct membrane compartment.

We are investigating the transport of the major light-harvesting chlorophyll a/b-binding protein (LHCP) into the chloroplast. LHCP is an integral membrane protein of the thylakoids, where it binds chlorophyll to maximize photosynthetic efficiency. LHCP is synthesized as a precursor in the cytoplasm (Schmidt et al., 1981) with an N-terminal transit peptide of 34–37 amino acids (Cashmore, 1984; Dunsmuir, 1985; Lamppa et al., 1985; Chitnis et al., 1986) that facilitates its import into the chloroplast. The precursor of LHCP (pLHCP) has been found in the thylakoid membranes after in vitro import (Chitnis et al., 1986, 1987), and inserts into isolated thylakoids in an ATP-dependent manner (Cline, 1986; Chitnis et al., 1987). One prediction from these results is that LHCP's transit peptide may also play an essential role in determining thylakoid localization.

In order to clarify further the function of the LHCP transit peptide and to distinguish between the steps of chloroplast import and intraorganellar routing, two questions were addressed. Can the integral membrane LHCP be mobilized into the chloroplast by the transit peptide of the small subunit (S) of ribulose-1,5-bisphosphate carboxylase/oxygenase, a stromal protein; and if so, what is the mature protein's fate after import and processing? Neomycin phosphotransferase, a soluble protein, is imported and found in the stroma when linked to the S transit peptide (Schreier et al., 1985; Van den Broeck et al., 1985; Kuntz et al., 1986; Wasmann et al., 1986). The transit peptide of ferredoxin is also able to direct proteins into the stroma, where ferredoxin is normally located. Even plastocyanin, a thylakoid lumen protein, was directed to the stroma when fused to the ferredoxin transit peptide (Smeekens et al., 1986, 1987). On the other hand, proteins transported into the chloroplast by the plastocyanin transit peptide were not efficiently transferred to the thylakoid lumen, but were found largely in the stromal compartment (Smeekens et al., 1986, 1987), suggesting that the transit peptide alone is not always sufficient for directing a protein to an inner compartment of the chloroplast.

In this study, I show that LHCP fused to the S transit peptide is successfully imported into pea chloroplasts and processed. Hence, even an integral membrane protein may be translocated by the transit peptide of a stromal protein. Most significantly, after import, LHCP did not remain in the stroma, but was inserted into the thylakoid membranes independent of its cognate transit peptide. These results provide evidence that the mature protein contains the signal for intraorganellar routing and, consequently, thylakoid localization.

EXPERIMENTAL PROCEDURES

Plant Materials—Pea (Pisum sativum) plants were grown in a greenhouse and transferred to a growth chamber (27 °C, cool fluorescent lights) 2 days before harvesting; usually 6 days after germination. Plasmid Construction—A wheat genomic clone (whAB1.6) coding for pLHCP, previously characterized (Lamppa et al., 1985), was inserted into transcription vector SP65 as described (Lamppa and Abad, 1987) to produce the construct SP65-pLHCP (originally SP6-whAB1.6). A cDNA sequence (Broglie et al., 1983) coding for the wheat small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase precursor (pS) was released from pBR322 with PstI and also inserted into SP65 in a 5′-to-3′ orientation, yielding the plasmid SP65-pS. To make the hybrid precursor, SP65-pS was digested first with BstI, which cuts 12 bases 3′ to the transit peptide sequence, producing a blunt end fragment. This was followed by digestion with HindIII to remove mature S. A fragment coding for mature LHCP was isolated from SP65-pLHCP by linearizing the plasmid with Hgal, which cuts immediately 5′ to the transit peptide-mature protein junction, and treated with mung bean nuclease (1-2 units/μg of DNA; Pharmacia LKB Biotechnology Inc.) to produce a blunt end in the correct reading frame. HindIII, which cuts about 550 nucleotides downstream of the LHCP stop codon, was used to release a 1.3-kilobase pair fragment coding for mature LHCP. The fragment was inserted by a directed ligation into plasmid SP66-pS that had been prepared with BstI and HindIII, and the chimeric construct was used to transform Escherichia coli (JM83). The insertion of the LHCP fragment produced a new FokI site at the hybrid precursor transit peptide-mature protein junction, which was used to confirm the correct codon fusion of the S transit peptide and mature LHCP sequences.

In Vitro Transcription, Translation, and Import Reactions—Plasmid DNAs were linearized with HindIII and transcribed in vitro using...
SP6 polymerase following the instructions of the supplier (Promega Biotech). The RNA (0.2 μg) was translated in a 35-μl wheat germ lysate reaction with [35S]methionine (Amersham Corp.). Pea chloroplasts were prepared, and import reactions were carried out essentially as described by Bartlett et al. (1982) and modified for smaller sample size (Lamppa and Abad, 1987). To separate the thylakoid and envelope membranes after import, the chloroplasts were lysed in 10 mM Hepes/KOH, pH 8, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride for 30 min on ice and loaded onto a gradient of steps of 0.4, 1.1, and 1.5 M sucrose made in Hepes/KOH, pH 8, as described (Cline, 1986). After centrifugation at 145,000 × g for 90 min, the envelopes and thylakoids were recovered from the 0.4–1.1 and 1.1–1.5 M sucrose interfaces, respectively. The envelopes were diluted 10-fold in 10 mM Hepes/KOH and pelleted at 145,000 × g for 1 h to remove the soluble phase. The thylakoids were also washed in 10 mM Hepes/KOH and pelleted at 16,000 × g for 15 min. Extraction of membrane fractions with 0.1 N NaOH was carried out as described (Schmidt et al., 1981). Samples were prepared for SDS-polyacrylamide gel electrophoresis as previously (Lamppa and Abad, 1987).

RESULTS AND DISCUSSION

A wheat cDNA sequence (Broglie et al., 1983) that codes for the S transit peptide was linked to a wheat genomic sequence coding for LHCP (Lamppa et al., 1985) previously inserted 3' to the SP6 promoter for in vitro transcription (see "Experimental Procedures"). The chimeric construct codes for a hybrid precursor that contains the proteolytic processing site Cys-Met from pS and the first four amino acids of mature S linked to mature LHCP. The processing site for pLHCP has not been definitively established; however, it most probably occurs between the Asn-Met or Met-Arg residues beginning 33 amino acids from the N terminus of the precursor. In this construct, the Met-Arg sequence was maintained (see Fig. 1).

RNA generated from the chimeric construct was translated in a wheat germ lysate, and the products were incubated with pea chloroplasts. After incubation, the chloroplasts were either directly lysed or pretreated with thermolysin to degrade proteins on the exterior (Cline et al., 1981; Joyard et al., 1983) and then separated into membrane and soluble fractions for SDS-PAGE. Wheat pLHCP and pS were also synthesized in vitro in the wheat germ lysate using RNA that was synthesized from their respective genes cloned into the SP6 transcription vector. The transport of pLHCP and pS into pea chloroplasts was assayed along with the hybrid precursor. Pea chloroplasts imported and processed both wheat pLHCP and pS, and the mature forms were resistant to thermolysin treatment of the organelles before lysis. Two forms of mature LHCP were localized to the membrane fraction, presumably the thylakoids (Fig. 2A, lanes 2 and 3), as observed previously (Kohorn et al., 1986; Lamppa and Abad, 1987); and S was found only in the soluble phase, the stroma (Fig. 2C, lanes 4 and 5). Usually, wheat pS was less efficiently transported into pea chloroplasts than wheat pLHCP (data not shown).

Following an import reaction using the hybrid precursor, the membrane and soluble fractions of the chloroplasts contained the precursor; however, in both fractions, most of it was sensitive to thermolysin, indicating that it represented protein on the surface of the organelle (Fig. 2B, lanes 2 and 3). In the membrane fraction, an additional, lower molecular weight band was found that migrated during SDS-PAGE with mature LHCP made in vivo (identified on Coomassie Blue-stained gels) and only slightly behind the products (25 and 26 kDa) of in vitro import and processing of radiolabeled pLHCP (Fig. 2A, lanes 2 and 3). The proteins in this band were resistant to pretreatment of the chloroplasts with thermolysin, demonstrating that they were associated with membranes within the chloroplast. No cleaved products were found in the soluble fractions after pretreatment with thermolysin. Two processed forms of mature LHCP were not readily resolvable as observed upon the import of pLHCP (Fig. 2A; see also Lamppa and Abad, 1987), but rather the hybrid precursor gave rise to multiple processed forms that ran as a relatively diffuse band. One possibility is that both cleavage sites of the parent precursor polypeptides, pLHCP and pS, were used and also other cleavages occurred due to the modifications at the hybrid precursor transit peptide-mature protein junction. In studies by others (Kuntz et al., 1986; Reiss et al., 1987), removal of cysteine and methionine at the cleavage site of pS did not prevent cleavage, suggesting that the sequence determinants for processing are distinct from the final site cleaved.

To determine if mature LHCP transported into the chloroplast by the S transit peptide was localized in the thylakoids or, alternatively, was associated within a thermolysin-resistant, envelope membrane component, sucrose step gradients were used to separate these two membrane systems after an import reaction. As a control, an aliquot of the import reaction was analyzed in the usual manner. Fig. 3 (lanes 2 and 3) shows the preferential localization of LHCP in the membranes. The remainder of the sample was hypotonically lysed, loaded onto a sucrose gradient, and centrifuged. Mature

![Fig. 1. Structure of hybrid precursor at transit peptide-mature protein junction.](https://example.com/fig1.png)

**Fig. 1. Structure of hybrid precursor at transit peptide-mature protein junction.** The chimeric DNA construct encoding the hybrid is described under "Experimental Procedures." The amino acid sequences are given in single-letter code of the N termini of pLHCP (Lamppa et al., 1985), the hybrid precursor, and pS (Broglie et al., 1985), which include the transit peptide and first 10 amino acids of each mature protein. The arrowheads point to the cleavage sites of the precursors. The pLHCP cleavage site is based on the evidence from peptide analysis of the N terminus of mature LHCP (Mullet, 1983). The open circles above the letters indicate the positions of serine or threonine, and the basic amino acids are indicated by plus signs.

![Fig. 2. Import of wheat hybrid precursor by pea chloroplasts.](https://example.com/fig2.png)

**Fig. 2. Import of wheat hybrid precursor by pea chloroplasts.** RNA coding for pLHCP, the hybrid precursor, and pS was translated in vitro in the wheat germ lysate, and the products (lanes 1) were incubated with isolated chloroplasts for 40 min. The chloroplasts were then either untreated (lanes 2 and 4) or treated (lanes 3 and 5) with thermolysin (subscript t) before organelle lysis. The chloroplast lysate was separated into membrane (M; lanes 2 and 3) and soluble (S; lanes 4 and 5) fractions by centrifugation before preparation for SDS-PAGE analysis. A, import of pLHCP; B, import of the hybrid precursor; C, import of pS. The arrow point to the processed precursor. A schematic of each precursor is shown at the bottom.
The hybrid precursor synthesized in vitro (Tr; lane 1) was incubated with pea chloroplasts; and after the reaction, one-fifth of the sample was lysed in 1 mM phenylmethylsulfonyl fluoride and separated into membrane (M; lane 2) and soluble (S; lane 3) fractions. The remaining chloroplasts were gently pelleted and lysed in 10 mM Heps/KOH, pH 8, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and held on ice for 30 min. The sample was then centrifuged over a sucrose step gradient, and envelope and thylakoid fractions were recovered (see "Experimental Procedures"). For direct comparison, the entire thylakoid (T; lane 4) and envelope (E; lane 5) fractions from the import reaction recovered from the gradient were analyzed.

LHCP sediments only with the thylakoid fraction and not with the envelopes (Fig. 3, lanes 4 and 5). The hybrid precursor, on the other hand, was found in the envelope fraction as expected since the organelles were not pretreated with thermolysin.

To establish if LHCP was inserted into the thylakoid membranes after import of the hybrid precursor, the membranes were extracted with 0.1 N NaOH to remove peripherally associated proteins (Steck and Yu, 1973; Schmidt et al., 1981). Fig. 4A shows the total membrane (lane 2) and soluble (lane 3) products of the import reaction, the 0.1 N NaOH membrane residue (lane 4), and the extractable proteins (lane 5). A parallel experiment was carried out for pLHCP (Fig. 4B). As shown, mature LHCP associated with the membranes was resistant to extraction with 0.1 N NaOH when either the hybrid or authentic precursor was used in the import reaction. Mature LHCP was also resistant to trypsin digestion in both cases.²

I conclude from these experiments that the small subunit transit peptide is capable of transporting the integral membrane LHCP into the chloroplast. Although the transit peptides of LHCP and S from wheat are different in length and amino acid sequence, both are basic and rich in threonine and serine (Broglie et al., 1983; Lamppa et al., 1985). The similarities are apparently sufficient to make LHCP and the S transit peptide compatible for import. My most important observation is that, after import, linkage to the S transit peptide did not retain mature LHCP in the stroma, nor did the absence of LHCP’s own transit peptide prevent thylakoid insertion. This is in contrast to the fate of plastocyanin when transported into the chloroplast by the transit peptide of the stromal protein ferredoxin. Once cleaved from the ferredoxin transit peptide, plastocyanin did not move into the thylakoid lumen where it normally functions, but instead it remained in the stroma (Smeekens et al., 1986). It has been suggested that a region of the plastocyanin transit peptide, a potential thylakoid transfer domain, is necessary for its successful translocation into the lumen (Smeekens et al., 1987).

In the hybrid protein used in this study, the transit peptide of pLHCP was completely removed and replaced with the S sequence. Thus, it appears that mature LHCP contains the structural properties that direct its thylakoid localization. I cannot exclude the possibility that any transit peptide with the features described above will participate in this process, but the exchangeability of the transit peptide demonstrates that it does not provide the routing information for LHCP. Recently, a soluble stromal factor has been identified that is necessary for in vitro insertion of pLHCP into the thylakoids (Chitnis et al., 1987), and one hypothesis is that this factor interacts with specific, as yet unidentified domains of the mature protein. Mutation analysis suggests that two regions of importance for membrane localization may be in the carboxy-proximal terminus of LHCP (Kohorn et al., 1986).

In conclusion, I provide evidence that LHCP does not require its cognate transit peptide for intraorganelar routing upon import or for thylakoid membrane insertion. Further studies are now required to define precisely the domains of mature LHCP required for these functions.

²G. K. Lamppa, unpublished results.
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