A Soluble Protein Factor is Required in Vitro for Membrane Insertion of the Thylakoid Precursor Protein, pLHCP

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ABSTRACT

The precursor to the light-harvesting chlorophyll a/b protein of photosystem II can insert into isolated thylakoid membranes if reaction mixtures also contain ATP and a soluble extract of chloroplasts. Optimization of this insertion process and the initial characterization of the soluble chloroplastic component are presented. With a fixed amount of precursor, maximum integration rates occurred during the first 30 minutes at pH 8.0 and 30°C when the soluble chloroplast extract was increased eight-fold over the stoichiometric amount. Under these conditions, insertion was routinely about 60% of that which occurred during import into intact chloroplasts. Integration also increased virtually linearly with increasing amounts of precursor. However, assays revealed that at least 40% of the in vitro-synthesized pLHCP was pelletable and inactive. The soluble chloroplastic component exhibited characteristics expected of a protein. It was inactivated by heat, protease, and N-ethylmaleimide, but was insensitive to ribonuclease. The soluble component migrated on a Sephacryl S-200 gel filtration column as a single peak with a $M_r$ of approximately 65,000. The proteinaceous nature of this factor suggests a similarity to soluble factors required for protein transport/integration in other membrane systems.

Many thylakoid membrane proteins are synthesized on cytosolic ribosomes as soluble, higher mol wt precursors that must subsequently import into chloroplasts (13, 16, 23). Because the internal thylakoid membrane system is separated from the delimiting chloroplast envelope membranes by an aqueous stroma, the trans-envelope transport of these proteins is physically and temporally separated from their site of stable thylakoid integration. Details of the assembly of these proteins are virtually unknown. However, preliminary evidence suggests that the precursors translocate across both envelope membranes directly into the stroma and then travel to and integrate into the thylakoids as soluble transport intermediates. First, Smeeckens et al. (25) reported that the precursor to plastocyanin, a thylakoid luminal protein, was proteolytically processed to an intermediate-sized precursor that cofractionated with the soluble fraction of chloroplasts. Because of its 'in pathway' assembly characteristics, they proposed the above-mentioned pathway for plastocyanin assembly. Second, Cline (10) reported an intrachloroplasmic process for incorporating thylakoid precursor proteins by showing that pLHCP could stably and specifically integrate into isolated thylakoid membranes. This process requires thylakoids, ATP, and a soluble chloroplastic extract.

Soluble factors, RNA and/or proteins, necessary for the efficient translocation of proteins across or into membranes have been discovered in every translocation system studied in-depth (2, 12, 21, 27, 30). The present communication reports the initial characterization of this essential chloroplastic soluble component under optimized conditions for pLHCP integration. Preliminary characterizations indicate that the soluble component is a single proteinaceous factor that lacks a functional RNA.

MATERIALS AND METHODS

Materials. [3H]Leucine was purchased from New England Nuclear. Nigerinic and Miracloth were from Behring Diagnostics. SP6 polymerase and RNAsin were from Promega Biotech. Molecular biology grade diananosine triphosphate and ribonucleotide triphosphates were from Pharmacia. Plasmid AB80XD/4 (an SP6 derivative of psAB80 [6]) was a kind gift of Thomas Moore and Dr. Kenneth Keegstra. Thermolysin, Percoll, Mg-ATP, proteinase K, ribonuclease A, PMSF, N-ethylmaleimide, dithiothreitol, Sephadex G-25, and Sephacryl S-200 were purchased from Sigma. All other chemicals were reagent grade.

Preparation of Chloroplasts and Lysates. Intact chloroplasts were isolated from 13- to 18-d-old pea (Pisum sativum var Laxton's Progress 9) seedlings as described (10). Unless otherwise stated, chloroplast lysates were obtained from intact chloroplasts by resuspending chloroplast pellets to 0.5 mg Chl/mL in 10 mM Hepes/KOH (pH 8.0) and incubating on ice for at least 4 min before use. Extent of chloroplast breakage was monitored on Percoll cushions (10). Thylakoids were prepared from chloroplast lysates by centrifugation at 3,200g$_{max}$ for 10 min at 4°C. Soluble extract was prepared from the resulting supernatant by further centrifugation at 42,000g$_{max}$ for 30 min at 4°C. Postribosomal soluble extract was prepared from thylakoid-free lysates by centrifugation in a Beckman SW-50.1 rotor at 40,000 rpm for 30 min at 4°C (5). The resulting supernatant was used for analysis. Unless noted, reconstituted lysates were prepared by resuspending thylakoid-free lysates in 42,000g$_{max}$ soluble extract to a final concentration of 0.5 mg Chl/mL.

Preparation of Radiolabeled Precursor. Precursor LHCP mRNA was prepared by SP6 polymerase transcription of EcoRI linearized psAB80XD/4. The transcription protocol was essentially as described by Promega Biotech Bulletin 001 except that transcriptions included 0.5 mm diananosine triphosphate, 0.005% BSA, 0.5 units of RNasin and 0.4 units SP6 polymerase/μL of transcription buffer and were not DNase-treated before use. Messenger RNA was translated in the presence of [3H] leucine in a wheat germ translation system (9). Translations were subsequently diluted approximately six-fold and adjusted to import buffer (0.33 m sorbitol, 0.05 m Hepes/KOH, pH 8.0), containing 30 mM leucine. Postribosomal precursor was prepared.
by centrifuging the adjusted translation mix at 40,000 rpm for 30 min in a Beckman SW-50.1 rotor (5).

Assays for Import and Integration of pLHCP. Assays were carried out in 10 × 75 mm glass test tubes. Import assays were performed as described (10). Unless otherwise stated, lysate assays were initiated by combining 200 μL of chloroplast lysate (0.5 mg Chl/mL), 50 μL of 60 mM Mg-ATP in import buffer, and 50 μL of adjusted translation product. All assays, regardless of the initial lysis conditions, contained approximately 100 μg total Chl. The mixture was incubated at 25°C in a covered water bath for 20 min with manual shaking approximately every 10 min unless otherwise stated. Assays were terminated with 2 mL of ice-cold import buffer, containing 0.45 μg nigericin (10). After termination, membranes were recovered from lysate assays by centrifugation at 3,200gmax for 10 min at 4°C. Membranes were then either analyzed directly or treated with thermolysin as described (10). Thermolysin treatments were terminated by addition of 100 μL of 50 mM EDTA in import buffer and 500 μL of 5 mM EDTA in import buffer. Membranes were recovered by centrifugation at 3,200gmax for 10 min at 4°C.

Analysis of Translation Products and Recovered Membranes. Samples of translation products, recovered chloroplasts, and recovered membranes were dissolved in sodium dodecyl sulfate buffer and subjected to denaturing electrophoresis through 0.75 mm, 12.5% acrylamide gels (18). After electrophoresis, the gels were either Coomassie-stained (18) or were prepared for fluorography as described (10). X-ray film, without refreshing, was exposed to the dried gels. Radioactive bands were excised from the gels and the tritium-labeled proteins extracted with NCS tissue solubilizer (Amer sham Corp.) (29). The number of molecules of pLHCP, LHCP, and LHCP-degradation product (protease resistant LHCP) was computed from the dpm of the extracted band (10). Import assays are reported as the number of molecules of pLHCP imported per chloroplast, while for lysate assays, integration is reported as molecules of LHCP-degradation product per chloroplast equivalent. The number of chloroplasts per lysate assay sample was computed as the number of lysed chloroplasts present in the assay mixture, assuming 100% recovery of membranes following the assay. The number of chloroplasts was calculated from their protein content and from the number of chloroplasts per milligram of protein, which was typically about 6.5 × 107.

Miscellaneous. The effect of pH on integration was monitored by lysing intact chloroplasts as previously described in 10 mM buffer/KOH (either MES [pH 6.0 and pH 7.0], Hepes [pH 7.0, pH 7.5, pH 8.0, pH 8.5, and pH 9.0], or Tricine [pH 9.0 and pH 10.0]) and assaying with correspondingly buffered 60 mM Mg-ATP and precursor for 30 min.

RESULTS

Optimization of Integration. Before characterizing the soluble component, it was important to examine and adjust assay parameters to optimum conditions. Integration assays are routinely conducted by incubating in vitro-synthesized pLHCP with either chloroplast lysates or reconstituted lysates and ATP ("Materials and Methods"). Previous assay parameters of 10 mM Mg-ATP (pH 8), 25°C, and 60 min were intuitively set without investigating their effect on the integrating activity (10). Cline (10) did find that integration was most efficient under low osmotic and ionic conditions, i.e., 23 mM Hepes/KOH (pH 8.0), 0.11 M sorbitol. Therefore, the present optimization studies were performed using this buffer. In addition to the parameters of ATP concentration, temperature, time, and pH, the response of integration to the relative quantity of both radiolabeled precursor and wheat germ translation mixture was determined. Unless noted, each optimization study was performed as described ("Materials and Methods") and successive parameter optimizations were cumulative.

Stable membrane integration was monitored by the partial resistance of precursor to exogenous protease ("Materials and Methods"). Integral membrane proteins are afforded at least partial protection against proteolysis by the membrane bilayer, while extrinsic proteins are susceptible (1). In the case of pLHCP, precursor integrated into thylakoids has an apparent molecular mass of 33 kD as monitored by denaturing polyacrylamide gel electrophoresis (Fig. 1, lane 4). If thylakoids are treated with thermolysin, the remaining protected degradation product (LHCP-DP) has an apparent molecular mass of 25 kD (Fig. 1, lanes 5 and 7). If pLHCP is only extrinsically associated with thylakoids (nonintegrated), it is not protease resistant (Fig. 1, lane 9).

Results of the optimization studies are shown in Figure 2. Integration increased virtually linearly with time up to 30 min (Fig. 2A), after which the rate diminished rapidly. Therefore, assays were routinely terminated after 20 to 30 min ("Materials and Methods"). Integration was maximum at 30°C (Fig. 2B), 10 mM Mg-ATP (Fig. 2C), and pH 8.0 (data not shown). The pH optimum was relatively narrow, with only 43 and 49% as much precursor integrating at pH 7.0 and 9.0, respectively, as at pH 8.0. To avoid the precipitous drop in integration at temperatures above 30°C, assays were routinely conducted at 25°C. In assays conducted such that all parameters and assay components remained constant except for the number of precursor molecules, integration increased virtually linearly with increasing precursor (Fig. 2D). The highest precursor level in Figure 2D represents 80% of the pLHCP synthesized in a typical in vitro translation reaction, so it is doubtful that integration can be saturated via a wheat germ system. Still, all subsequent assays were performed with precursor levels that fell within the linear portion of the curve. Interestingly, the wheat germ translation mixture had a stimulatory effect on integration. Doubling the relative amount of translation mixture within an assay, while maintaining all

Fig. 1. Precursor integrates posttranslationally. Radiolabeled pLHCP (T) was prepared, then fractionated by ultracentrifugation into a soluble, postribosomal supernatant (S) and a pelletable component (P) as described in "Materials and Methods." Recovered membranes from lysates received either no treatment (lanes 4, 6, and 8) or were protease-treated (lanes 5, 7, and 9) before analysis by SDS-PAGE/fluorography ("Materials and Methods"). Intact chloroplasts from import assays were either repurified directly (lanes 10 and 12) or protease-treated (lanes 11 and 13) before repurification on 35% Percoll cushions (10). Repurified, intact chloroplasts were then analyzed similarly as lysates. Assays performed with either total, soluble or pelletable precursor received approximately 4650, 5800, and 3300 molecules of precursor per chloroplast (chloroplast equivalent), respectively.
other components (including pLHCP) constant, increased integration by 50% (data not shown). This enhancement was not further investigated, but for quantitative purposes the translation mixture was held constant within experiments.

Integration Occurs Posttranslationally with Soluble pLHCP. It is implicit in the proposed 'soluble pathway' mechanism that integration occurs posttranslationally with soluble precursors (see introduction). The pLHCP used in our assays is synthesized in wheat germ translation extracts in the presence of [3H]leucine for 60 min, after which the incorporation of radiolabel is terminated by transferring the translation to 0°C and diluting it about six-fold with unlabeled leucine. To investigate the implicit requirements, a terminated translation was centrifuged to remove both ribosomes and any insoluble pLHCP. Approximately 40% of the pLHCP was recovered in the pellet. Integration assays were subsequently conducted with soluble pLHCP (supernatant fraction) and pelleted pLHCP that was resuspended in a mock translation supernatant (Fig. 1). Although both soluble and pelleted pLHCP bound to thylakoids (Fig. 1, lanes 6 and 8), only the soluble pLHCP was integrated into the membrane (Fig. 1, lanes 7 and 9). Further, in parallel assays containing approximately the same total amount of pLHCP, twice as much integration occurred with soluble pLHCP than with unfractionated pLHCP (Fig. 1, lanes 5 and 7). Similar assays with intact chloroplasts showed that only soluble pLHCP could be imported (Fig. 1, lanes 11 and 13). The observation that at least 40% of the pLHCP produced in a wheat germ extract is inactive explains, in part, the relatively low percentage of precursor that is either imported (12–15%) or integrated (3–10%).

These results also demonstrate that integration in the in vitro assay occurs posttranslationally and does not require any precursor association with ribosomes. We have verified that integration is posttranslational during standard assays, i.e., unfractionated pLHCP, by conducting integration assays with cycloheximide-terminated pLHCP translation mixtures, which yield essentially the same amount of integration as translation mixtures terminated by our standard procedure (data not shown). Other experiments have shown that cycloheximide causes an immediate termination of pLHCP translation (data not shown).

Soluble Component is Limiting under Standard Assay Conditions. The original assay parameter selections were quite good, but under these conditions, as reported (10), lysate assays usually achieved only 30% of the integration that occurred in comparable import assays with intact chloroplasts. However, lysates were routinely prepared at 0.5 mg Chl/mL and assayed at 0.3 mg Chl/mL. Such preparations represent a 120-fold dilution of the chloroplastic contents (8). To assess the effect of such a dilution on pLHCP integration, assays were conducted with varying amounts of soluble extract, ranging from no extract to one-fifteenth (8 relative extract units) the concentration of that in an intact chloroplast (Fig. 3). A low, variable level of integration occurred in the absence of soluble extract. Inclusion of soluble extract resulted in integration that increased with increasing extract up to 8 relative units, where the response was nearly saturated. Under these conditions the level of integration is greatly increased over that achieved under standard conditions. In the experiment shown in Figure 3, more than twice as much
SOLUBLE FACTOR REQUIRED FOR pLHCP INTEGRATION

Fig. 3. Level of soluble extract determines the extent of integration. Soluble extract was prepared as described in "Materials and Methods," except that chloroplasts were lysed at 4 mg Chl/mL. An aliquot of the resulting soluble fraction was then serially diluted with 10 mm Hepes/KOH (pH 8.0) to give a 32-fold dilution range, i.e. 0.25 to 8 relative extract units, with 1 unit extract equivalent to lysing intact chloroplasts at 0.5 mg Chl/mL. Each dilution in the series was reconstituted with freshly prepared thylakoids at a final concentration of 0.5 mg Chl/mL and each reconstituted lysate assayed for integration ("Materials and Methods"). Each assay received approximately 5000 molecules of pLHCP per chloroplast equivalent.

pLHCP was integrated with 8 relative extract units than with 1 unit. However, a greater than threefold stimulation has been observed. These results show that under standard assay conditions, soluble extract is limiting. More importantly, they indicate that the soluble factor can be quantitated by its ability to stimulate integration. For convenience, we continued to prepare lysates and soluble extract from chloroplasts at 0.5 mg Chl/mL.

Soluble Component is Resistant to Ribonuclease but not Protease. Early experiments established that the soluble chloroplastic factor was probably macromolecular in nature, being heat labile, relatively large, and ammonium sulfate precipitable; i.e. approximately 30 and 97% of the integrating activity was lost when soluble extract was heated for 10 min at 37 and 100°C, respectively, while a variable 69 to 75% of the activity was recovered with the void volume off a Sephadex G-25 column (exclusion limit 5000 D) and 34% of the activity was recovered with a saturated ammonium sulfate pellet (no activity was recovered with the supernatant). Since soluble factors necessary for the efficient translocation of proteins into and across membranes in other systems have been reported as being RNA and/or proteins (2, 12, 21, 27, 30), the chloroplastic component was tested for its sensitivity to both ribonuclease and protease.

Thylakoids and postribosomal soluble extract were prepared ("Materials and Methods") and treated independently with either active or deactivated ribonuclease before assaying with postribosomal precursors. Postribosomal extract and precursor were used as a precaution because it was reported that ribonuclease treatment of cell-free translation mixtures containing ribosomes resulted in the inhibition of protein import into mitochondria (5). If the ribosomes were first removed, ribonuclease treatment of translation mixtures had no effect upon mitochondrial protein import.

Treatment of soluble extract (Fig. 4, bars 2 and 3) and thylakoids (Fig. 4, bars 4 and 5) with inactive and active ribonuclease, respectively, had no significant effect upon integration. Control experiments demonstrated that the ribonuclease used was active under these conditions (data not shown). The experiment shown in Figure 4 was conducted with 0.1 mg/mL RNase. However, RNase was without effect on the activity of the soluble component even when up to 1.0 mg/mL was used (data not shown). Similarly, micrococcal nuclease at up to 1000 units/mL was without effect on soluble component activity (data not shown). Obviously, if RNA does play a functional role in integrating pLHCP into thylakoids or chloroplast lysates, then it is inaccessible to ribonuclease. It is also clear that the soluble component is truly soluble, remaining with the postribosomal supernatant.

A similar experiment, testing the possible proteinaceous nature of the soluble component, assayed thylakoids and soluble extract that were independently treated with either deactivated or active proteinase K prior to reconstitution (Fig. 5). Soluble extract and thylakoids alternately treated with deactivated proteinase K (Fig. 5A, bars 2 and 4) integrated pLHCP at levels 100% and 57% of control (Fig. 5A, bars 1), respectively. The control consisted of mock-treated, reconstituted lysates. When subfractionated components were alternately treated with active proteinase K, the protease subsequently deactivated and the components reconstitute, integration of precursor was almost abolished; less than 5% of control was achieved by each (Fig. 5A, bars 3 and 5). Clearly, this demonstrates two points: first, that the soluble component is proteinaceous, and second, that precursor assembly is also dependent upon thylakoid membrane proteins.
Since thylakoid membrane proteins are sensitive to protease and are essential for integration, it was important to monitor the degree of PMSF deactivation of proteinase K ("Materials and Methods"). Protease deactivation was demonstrated in two manners. First, BSA and radiolabeled pLHCP were independently incubated with deactivated and active proteinase K under identical conditions as extract and thylakoids. Both were resistant to digestion, as monitored by their electrophoretic profiles, when treated with deactivated protease, but were degraded when treated with active protease (data not shown). Second, Coomassie-stained electrophoretograms ("Materials and Methods") of postassay thylakoids (Fig. 5B) showed that membranes incubated with deactivated proteinase K (lane 6) and membranes reconstituted with protease-treated extract (lane 4) had protein profiles identical to nontreated thylakoids (lanes 2, 3); whereas, proteinase K treated-thylakoids showed a drastically altered banding pattern (lane 1).

**Soluble Factor is Sensitive to N-ethylmaleimide.** In another similar experiment, soluble extract and thylakoids were alternately treated with N-ethylmaleimide in either the absence or presence of excess dithiothreitol, reconstituted, and assayed for integrating activity (Fig. 6). Assays conducted with the N-ethylmaleimide-treated soluble extract (Fig. 6, bar 2) showed an 83% reduction in integration of precursor as compared to mock-treated lysates (Fig. 6, bar 1), while assays conducted with similarly treated thylakoids showed an increase (Fig. 6, bar 4). It is uncertain why treating thylakoids with N-ethylmaleimide stimulates integration. N-Ethylmaleimide treatment of either soluble extract or thylakoids in the presence of excess dithiothreitol prevented the respective inhibitory and stimulatory effects observed with N-ethylmaleimide alone (Fig. 6, bars 3 and 5). Clearly, the soluble component is not only proteinaceous, but has a sulfhydryl group that renders it non-functional when blocked by N-ethylmaleimide.

**Molecular Weight Analysis.** In order to estimate the molecular size of the factor, soluble extract was fractionated on a Sephacryl S-200 gel filtration column. SDS-PAGE analysis of the fractions (Fig. 7, upper panel) demonstrates the degree of fractionation achieved. The soluble factor eluted as a single peak of activity with an $M_r$ of 65,000 (Fig. 7, lower panel, inset). The small rise in activity near the included volume of the column was not reproducible from experiment to experiment. However, the inhibition of activity (below background) in the excluded volume of the column was reproducible. The width of the peak was similar to that of the closest protein standard (BSA), indicating that the soluble factor is a single species. Unfortunately, the recovery of activity from the S-200 column was relatively low. In the experiment shown in Figure 7, 20% of the activity applied to the column was recovered in the large peak. In other experiments, as little as 11% of the activity was recovered. It is possible that upon fractionation of the stroma, the soluble factor becomes unstable or more susceptible to proteases. However, we presently cannot rule out the possibility of an additional factor(s) that is inactive by itself, but capable of stimulating the activity of the $M_r$ 65,000 factor.

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**Fig. 5. Soluble component is protease sensitive.** Soluble extract and thylakoids were prepared ("Materials and Methods") and treated independently with either active or PMSF-deactivated proteinase K (50 µg/mL) at 4°C for 30 min. Active proteinase K was deactivated after treatment. Proteinase K was deactivated at 4°C for 30 min with the addition of freshly prepared PMSF (100 mM in ethanol) to a final concentration of 2.5 mM. Thylakoids were treated at 0.5 mg Chl/mL in 10 mM Hepes/KOH (pH 8.0), pelleted at 3200g, and resuspended and pelleted as before. A, Treated thylakoids were reconstituted with untreated soluble extract, and vice versa, then assayed and analyzed for integration ("Materials and Methods"). 1, Reconstituted lysate, mock treatment (110 molecules pLHCP integrated per chloroplast equivalent); 2, soluble extract plus deactivated protease; 3, soluble extract plus active protease; 4, thylakoids plus deactivated protease; 5, thylakoids plus active protease. Each assay received approximately 3000 molecular precursor per chloroplast equivalent. B, Coomassie-stained SDS-PAGE of thylakoid membrane samples. Lanes: 1, thylakoids treated with proteinase K; 2, untreated thylakoids; 3, thylakoids recovered from the control (mock treatment) assay; 4, thylakoids recovered from the assay of deactivated proteinase K-treated extract; 5, thylakoids recovered from the assay of proteinase K-treated extract; 6, thylakoids recovered from the assay of deactivated proteinase K-treated membranes. Mol wt standard proteins were: catalase (57,000), aldolase (40,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,000), and hemoglobin (15,000).
SOLUBLE FACTOR REQUIRED FOR pLHCP INTEGRATION

FIG. 6. Soluble component is sensitive to N-ethylmaleimide. Soluble extract and thylakoids were prepared ("Materials and Methods") and treated independently with 1 mM N-ethylmaleimide either in the absence or presence of 2 mM dithiothreitol at 0°C for 15 min. N-Ethylmaleimide and dithiothreitol were freshly prepared in 10 mM Hepes/KOH (pH 8.0) (100 mM) before treatments. After 15 min, N-ethylmaleimide-treated components were adjusted to 2 mM dithiothreitol to scavenge any unreacted N-ethylmaleimide. Thylakoids were treated at 0.5 mg Chl/mL in 10 mM Hepes/KOH (pH 8.0), pelleted at 3200g_{av} for 10 min, then resuspended in soluble extract adjusted to 2 mM dithiothreitol. Treated thylakoids were reconstituted with untreated soluble extract and vice versa, then assayed and analyzed for integrations ("Materials and Methods"). 1. Reconstituted lystate, mock treatment (97 molecules precursor integrated per chloroplast equivalent); 2 and 3, soluble extract, N-ethylmaleimide-treated in the absence or presence of dithiothreitol, respectively; 4 and 5, thylakoids, N-ethylmaleimide-treated in the absence or presence of dithiothreitol, respectively; 6, reconstituted lystate, plus 2 mM dithiothreitol. Each assay received approximately 3000 molecules of precursor per chloroplast equivalent.

DISCUSSION

Two previous communications (10, 11) documented that pLHCP can insert into isolated thylakoid membranes, assume the correct bilayer orientation, and, under certain conditions, be proteolytically processed and assembled into the PSII Chl-protein complex. In the studies reported here, we have further investigated assay parameters of the insertion process and partially characterized one of the macromolecular components of the assembly apparatus. Results of these studies indicate that insertion activity is maximal under conditions thought to occur in an intact chloroplast. These include pH 8.2 to 30°C, and either exogenous ATP or, as recently shown (11), light. Integrating activity also increased as the concentration of soluble extract in assay mixtures approached that occurring within chloroplasts. Further, only fully synthesized, soluble pLHCP could insert. These findings indicate that the insertion apparatus characterized in these studies would be active under physiological conditions in vivo.

The soluble chloroplastic component necessary for pLHCP integration is proteinaceous, does not appear to contain a functional RNA moiety, and appears to be a single component as determined by gel filtration chromatography. Such a factor within chloroplasts was not totally unexpected as proteinaceous soluble factors within other systems have proven necessary for the efficient translocation of proteins into and across membranes. They have been documented in organisms as diverse as mammals (2, 26), yeast (30), and bacteria (12, 21) and have been shown necessary for both protein export (12, 21) as well as organellar import (2, 26, 30). The chloroplast factor differs from the best characterized of these factors, the SRP. It is not sensitive to ribonuclease, whereas SRP is a multimeric protein complex containing 7S RNA (27). Further, the chloroplast factor does not seem to associate with membranes or ribosomes as does SRP, but remains in the postribosomal supernatant (28). The chloroplast factor, at least in content, appears more similar to protease-sensitive, ribonuclease resistant soluble factors (2, 12, 21, 30). At the present, the stromal soluble factor is the only known localization factor that functions within an organelle.

Several of the reported soluble factors interact with the precursor to be transported or integrated (2, 12, 26). The presumed function of this interaction is to direct the precursor to the target membranes and/or render the precursor translocation competent (2, 12, 26). We think it unlikely that the chloroplast factor directs pLHCP to the thylakoid membrane because pLHCP extrinsically associates with thylakoids in the absence of soluble factor (10, 11). In fact, no condition that prevents integration, including absence of ATP and protease pretreatment of thylakoids, has been found where pLHCP did not bind to thylakoids. It is possible, however, that the chloroplast factor induces a pLHCP conformation necessary for integration either by unfolding, refolding (19, 22, 31) or posttranslationally modifying the precursor. The demonstration that pelletable pLHCP is not competent for integration reinforces the notion of a conformational constraint. Further, several posttranslational modifications of LHCP have been demonstrated including acylation (4), phosphorylation (3), association with pigments (1), and possibly palmitoylation (20). It is clear that proteolytic maturation of pLHCP is not required for insertion, but the potential involvement of other modifications has not been explored. In any case, elucidating the factor's role in integration requires its purification. Unfortunately, attempts to purify the factor by traditional procedures have so far proven unsuccessful, primarily due to inactivation of the factor by a variety of different conditions (unpublished observations of DR Fulsom and K Cline).

It is conclusive that an apparatus for assembling pLHCP into thylakoids exists within chloroplasts and, as we have shown here, that it consists of a soluble protein factor and exposed proteins on the thylakoid membrane. This apparatus is efficient, specific (10), and assembles pLHCP with apparently the same fidelity that occurs in vivo (11). Although there is no definitive evidence that this apparatus is used for pLHCP integration in vivo, several recent studies lend strong support to this notion. Chitnis et al. (7) showed that both the membranes and the soluble extract from barley etioplasts acquire the ability to integrate pLHCP during the developmental transition from etioplasts to chloroplasts, coinciding with the developing ability of the intact plastids to integrate imported pLHCP. In addition, evidence from Smeevens et al. (25) for the thylakoid luminal protein, plastocyanin, and from Hartl et al. (14, 15) for several cytosolically synthesized mitochondrial intermembrane space proteins indicate a 'soluble matrix' pathway for intraorganellar protein assembly. Hartl et al. (14, 15) have speculated that, subsequent to transport into the organelle matrix, these proteins are assembled by the conserved 'ancestral pathway' of the prokaryotic endosymbiont (17, 24), i.e. the pathway presently used for the assembly apparatus of organelle-encoded membrane proteins. In this regard, it will be interesting to learn if plastid-encoded thylakoid proteins utilize the same assembly apparatus as does pLHCP. The answer to this question must await further characterization of the components of the assembly apparatus and...
FIG. 7. Size-fractionation of soluble extract on Sephacryl S-200. A soluble extract was prepared as described in “Materials and Methods” by lysing intact chloroplasts at 4 mg Chl/Ml in 10 mm Hepes/KOH (pH 8), 1% ethylene glycol (v/v), followed by adjustment to 75 mM NaCl. A 3 mL aliquot was fractionated on a 2.5 x 35 cm Sephacryl S-200 column that was eluted with the same buffer at 15 mL/h. Fractions 14 through 39 (4.3 mL/fraction) were assayed for polypeptide composition by SDS-PAGE followed by Coomassie staining of the gel (upper panel of the figure) and for integrating activity (lower panel). The reconstituted lysates for integration assays were prepared by combining 150 μL of column fraction with 50 μL of freshly prepared thylakoids suspended at 2 mg Chl/mL in the same buffer. Assays were carried out as described in “Materials and Methods” for 30 min. Each assay received approximately 9000 molecules pLHCP/chloroplast. The plotted integration values were corrected for background integration (20 molecules/chloroplast) that occurred in the absence of added soluble extract. The protein standards used for mol wt calibration of SDS-PAGE were: phosphorylase b (94,000), BSA (66,000), catalase (57,000), aldolase (40,000), malate dehydrogenase (34,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,000), and hemoglobin (15,000). An unfractionated soluble extract is depicted in the leftmost lane of the gel (SE). The protein standards used to calibrate the Sephacryl S-200 column (lower panel, inset) were: BSA (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), and Cyt C (12,400). The arrow depicts the location of the soluble factor.

examination of assembly requirements for other thylakoid proteins.

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