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Stromal factor plays an essential role in protein integration into thylakoids that cannot be replaced by unfolding or by heat shock protein Hsp70

(soluble factor/molecular chaperone/translocation competence/membrane translocation)

JIANGUO YUAN, RALPH HENRY, AND KENNETH CLINE*

Plant Molecular and Cellular Biology Program, Horticultural Sciences Department, University of Florida, Gainesville, FL 32611

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ABSTRACT **The light-harvesting chlorophyll a/b protein (LHCP) is an integral thylakoid membrane protein. It is made in the cytosol as a precursor (pLHCP), imported into chloroplasts, and subsequently integrated into thylakoids. Integration of pLHCP into thylakoids requires a stromal protein factor that functions in part to maintain the solubility and integration competence of pLHCP. Recently, it was reported that unfolded pLHCP was sufficient for integration and that the stromal factor, identified as the plastid Hsp70, was required only to prevent pLHCP refolding [Yalovsky, S., Paulsen, H., Michaeli, D., Chitnis, P. R. & Nechushtai, R. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5616–5619]. Our studies, using more rigorous criteria for integration, show that unfolded pLHCP is not sufficient; stromal factor is an absolute requirement for integration. Furthermore, experiments with purified Hsp70 as well as Hsp70-depleted stromal extract demonstrate that Hsp70 is not the stromal factor. These results plus the finding that pLHCP diluted out of urea is relatively stable as a substrate for integration point to an additional role for the stromal factor in targeting and/or membrane translocation.**

Soluble factors necessary for efficient transport of proteins across or into membranes have been discovered in nearly every translocation system studied in depth. Many soluble factors such as the cytosolic heat shock protein Hsp70, the mitochondrial Hsp60, and the bacterial SecB, DnaK, and GroEL are molecular chaperones that function to prevent nonproductive reactions of preproteins such as premature folding (1–6), aggregation (7), or nonspecific membrane association (8). Certain soluble factors, such as SecB of bacteria and the signal recognition particle (SRP) of mammalian cells, also provide a targeting function. SecB facilitates preprotein targeting to the translocase via its affinity for SecA (8). SRP binds to signal peptide of nascent preproteins (9) and targets them to the endoplasmic reticulum by subsequent binding to the SRP receptor (10). Some soluble factors—e.g., the bacterial SecA (11)—also participate in the mechanisms of membrane translocation.

The light-harvesting chlorophyll a/b protein (LHCP) is nuclear-encoded and must cross the chloroplast envelope before being integrated into thylakoids. Integration of LHCP into thylakoids has been reconstituted *in vitro* and shown to require thylakoids, ATP, and an as yet unidentified stromal protein factor (12, 13). Both the intact precursor (pLHCP) and the mature-sized protein (LHCP) can serve as substrates for the reconstituted reaction (12, 14). Payan and Cline (15) showed that one function of the stromal factor is to maintain the solubility and integration competence of (p)LHCP (LHCP or pLHCP). The stromal factor accomplishes this by converting (p)LHCP into a large and soluble species, most likely a complex between (p)LHCP and part of the stromal factor. This putative complex has an estimated molecular mass of about 120 kDa. The ability of the stromal factor to convert pLHCP into the 120-kDa complex correlates with the ability of stromal protein to promote pLHCP integration, supporting the idea that complex formation is a step leading to thylakoid integration.

Recently, the precursor form of LHCP has been overexpressed in *Escherichia coli* and the purified pLHCP used *in vitro* to study the importance of soluble factors in protein import into chloroplasts or integration into thylakoids. First, Waegemann *et al.* (16) reported that urea-solubilized pLHCP was not competent for import; dialysis of pLHCP with soluble proteins was essential to obtain import competence. Second, Yalovsky *et al.* (17) reported that when urea-denatured pLHCP was directly diluted into the integration reaction, it was inserted into thylakoids in the absence of stromal extract (SE). Further, they reported that SE was required only if urea-denatured pLHCP was dialyzed prior to the integration reaction and that the plastid Hsp70 could replace SE in this reaction—i.e., Hsp70 is the stromal factor.

We have reexamined the soluble factor requirement(s) for pLHCP import and integration using purified *E. coli*-made pLHCP as well as *in vitro*-translated pLHCP. Our studies have shown that whereas unfolded pLHCP is efficiently imported into chloroplasts without cytosolic factors, its integration into thylakoids absolutely requires SE. Hsp70 could not replace SE in the integration reaction and depleting SE of Hsp70 did not impair its ability to support integration—i.e., Hsp70 is not the stromal factor. When considered with the essential nature of SE, the finding that pLHCP is surprisingly stable in aqueous solution indicates that the stromal factor most likely has an active role in the integration reaction.

MATERIALS AND METHODS

Materials. [^{3}H]Leucine was from DuPont/NEN. RNasin and SP6 RNA polymerase were from Promega. MgATP, thermolysin, fast-flow DEAE-Sepharose, C-8 linked ATP-agarose, protein A-Sepharose 4B, and Percoll were from Sigma. All other chemicals were reagent grade. Plasmid psAB80XD/4 is the *in vitro* expression plasmid for pLHCP (18). Plasmid pETHPLHCP is the *E. coli* overexpression plasmid for pLHCP (38). Several antibodies were used in this study. The antibody to LHCP has been described (15). Antibody to the *E. coli* DnaK was kindly provided by John McCarty and Caroline Donnelly (Massachusetts Institute of

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Abbreviations: Hsp70, 70-kDa heat shock protein; LHCP, light-harvesting chlorophyll a/b protein; pLHCP, precursor to LHCP; (p)LHCP, LHCP or pLHCP; SE, stromal extract; DTT, dithiothreitol; BSA, bovine serum albumin.

*To whom reprint requests should be addressed.

Technology, Cambridge). Antibody to tomato cytosolic Hsp70 was a gift of L. Nover (19).

Preparation of Radiolabeled Precursor, Chloroplasts, Lysates, Thylakoids, and SE. ^{3}H-labeled pLHCP was prepared either by *in vitro* transcription (13) and translation (12, 18) or by overexpression in *E. coli* in the presence of [^{3}H]leucine (38). Chloroplasts were isolated from 9- to 10-day-old pea (Laxton's Progress 9) seedlings as described (12, 20) and were resuspended in import buffer (50 mM Hepes/KOH, pH 8/0.33 M sorbitol). Lysates, thylakoids, and SE were prepared from isolated chloroplasts (13, 20). Lysates prepared at 0.5 mg/ml chlorophyll (Chl) were arbitrarily referred to as 1× lysate and the SE resulting from such lysate as 1× SE (≈3 mg of protein per ml).

Purification of Hsp70 from SE and Preparation of Anti-Hsp70 Antibody. Stromal Hsp70 was purified according to Welch and Feramisco (21) as follows. Stromal protein (100 mg) in buffer A [20 mM Hepes/KOH, pH 8/20 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol (DTT)] was loaded at 1 ml/min onto a 10-ml fast-flow DEAE-Sepharose column. After the column was washed at 2 ml/min with 30 ml of buffer A, the bound proteins were eluted at 1 ml/min with a 50-ml, 20–500 mM KCl gradient. The fractions containing Hsp70 at ≈250 mM KCl were pooled, diluted 5-fold with buffer A, and applied at 10 ml/hr to a 5-ml ATP-agarose column that had been equilibrated with buffer A. The column was sequentially washed with 10 ml of buffer A, 20 ml of 0.5 M KCl in buffer A, and 20 ml of buffer A and was then eluted with 30 ml of buffer A containing 3 mM ATP. The resulting preparation was about 70% Hsp70 and 25% Hsp60 as determined by densitometry of Coomassie blue-stained gels. The identity of Hsp70 was confirmed by specific reaction with antibody against *E. coli* Hsp70/DnaK (*Results*). Hsp70 was further purified by SDS gel electrophoresis, electroeluted from gel bands, and used for antibody production in rabbits.

Depletion of Hsp70 from SE. Protein A-Sepharose 4B (1-ml aliquot) was washed (15), suspended in 3 ml of 10 mM Hepes/KOH, pH 8, and then mixed with 3 ml of either 2% bovine serum albumin (BSA), preimmune serum, or anti-Hsp70 serum. The mixture was incubated overnight at 4°C and the supernatant was removed by centrifugation at 500 × *g* for 2 min. After three washes (5 ml each) with column buffer (25 mM Hepes/KOH, pH 8/50 mM KCl/10 mM $MgCl_2$), protein A-Sepharose matrices were transferred into syringe columns with glass fiber filter supports. SE (2 ml of 1× SE) was applied to each column and allowed to pass through after 15 min of incubation. The flow-through was reapplied to the column two more times in a similar fashion and was finally recovered from the column by centrifugation at 500 × *g* for 3 min.

Assays for Import, Integration, and Soluble Complex Formation. Import assays were conducted for 10 min as described (12, 20) except that *E. coli*-made pLHCP was used instead of *in vitro*-translated pLHCP. Assays were started by adding urea-denatured pLHCP to the assay mixture as described in the figure legends. Unless otherwise specified, import reactions (300 μl) contained chloroplasts equivalent to 0.33 mg of Chl per ml, 50 mM Hepes/KOH (pH 8), 0.33 M sorbitol, 10 mM MgATP, 2 mM DTT, 0.2 M or less urea, and ≈0.2 μM pLHCP. Integration assays were performed with *E. coli*-made pLHCP and with *in vitro*-translated pLHCP for 30 min essentially as described (12, 18). Integration reactions (300 μl) with *E. coli*-made pLHCP received thylakoids equivalent to 0.33 mg of Chl per ml, 9 mg of stromal protein per ml, 17 mM Hepes/KOH (pH 8), 55 mM sorbitol, 10 mM MgATP, 1 mM DTT, 0.2 M or less urea, and ≈0.2 μM pLHCP. To minimize microcentrifuge tube-adsorbed pLHCP, washed thylakoids recovered from integration assays were transferred to new tubes before analysis. Assays for soluble complex formation were performed according to Payan and Cline (15).

Sample Analyses. Samples recovered from the above assays were subjected to electrophoresis on 12.5% SDS/polyacrylamide gels (22) and fluorography (23). About 10% of the chloroplasts or thylakoids recovered from each assay were loaded per gel lane. Quantification of import or integration was accomplished by scintillation counting of radiolabeled proteins extracted from excised gel bands (12, 24).

Miscellaneous Methods. Chl concentrations were determined according to Arnon (25). Protein assays were performed by the BCA method (Pierce) for samples without DTT or by the Bradford method (26) for samples with DTT using BSA as a standard. Immunoprecipitation and immunoblotting were carried out as described (15).

RESULTS

Stromal Components Are Absolutely Essential for Integration of pLHCP into Thylakoids. pLHCP was produced by overexpression in *E. coli*. The overexpressed pLHCP was sequestered in inclusion bodies. Isolation of inclusion bodies yielded pLHCP that was 90–95% pure as determined by densitometry of Coomassie blue-stained gels. The specific radioactivity of *E. coli*-made pLHCP was 400,000–800,000 dpm/μg of protein. When purified pLHCP was denatured in 8 M urea and then directly diluted into reaction mixtures, it immediately adopted a form that was competent either for import into chloroplasts or for integration into thylakoids (Fig. 1).

Purified pLHCP was able to import into isolated chloroplasts in the absence of any soluble factors as long as energy (ATP or light) was provided (Fig. 1*B*). The *E. coli*-made substrate was imported as efficiently as *in vitro*-translated pLHCP; up to 15% of the added pLHCP was imported during the 10-min incubation. Addition of cytosolic components—i.e., wheat germ extract or reticulocyte lysate—did not

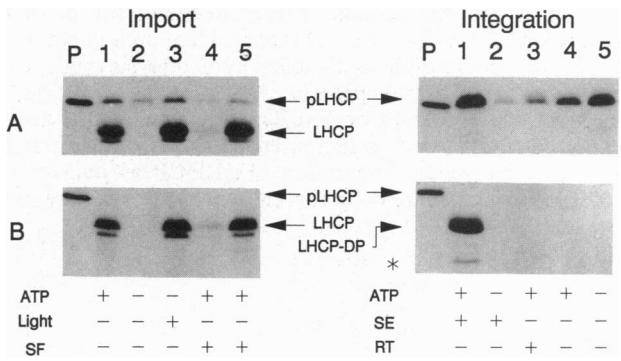

FIG. 1. Soluble factors are not required for import of purified pLHCP into chloroplasts, whereas unfolded pLHCP still requires SE for integration into thylakoids. *E. coli*-made pLHCP was dissolved in 8 M urea/8 mM DTT at room temperature for 4 hr and then directly diluted into assays for import into chloroplasts or integration into thylakoids. Import assays were carried out either in dark with 10 mM ATP (lanes 1, 4, and 5) or without ATP (lane 2), or in light (≈70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with no added ATP (lane 3). Some assays also received soluble factors (SF)—i.e., wheat germ protein (lane 4, 1 mg) or rabbit reticulocyte protein (lane 5, 3 mg). Integration assays were carried out in dark with 10 mM ATP (lanes 1, 3, and 4) or without ATP (lanes 2 and 5). Some assays also received 3 mg of protein of SE (lanes 1 and 2) or rabbit reticulocyte lysate (RT, lane 3). Recovered chloroplasts and thylakoids were either extracted with 0.1 M NaOH (*A*) or treated with thermolysin (*B*). Gels were loaded on an equivalent Chl basis (4.5 μg per lane). Lanes P, purified pLHCP. LHCP-DP, a diagnostic protease degradation product of correctly assembled LHCP or pLHCP (18, 27, 28). Marker (*) points at a protease degradation product previously characterized as inserted but resulting from incompletely assembled pLHCP (29).

stimulate the import of purified pLHCP. On the contrary, elevated quantities of wheat germ extract inhibited import of *E. coli*-made pLHCP (Fig. 1, lane 4) as well as *in vitro*-translated pLHCP (unpublished results). Waegemann *et al.* (16) previously reported that urea-denatured pLHCP was not competent for import unless it was first dialyzed with cytosolic factors (leaf extract). It is apparent from Waegemann *et al.* (16) that cytosolic factors can be helpful to pLHCP import under certain conditions. However, the results in Fig. 1 demonstrate that they are not essential.

In contrast, integration of purified pLHCP into isolated thylakoids absolutely required the presence of SE in addition to ATP (Fig. 1*B*). Titration of the stromal dependence showed that at least 2.5 mg of stromal protein per ml was necessary to obtain appreciable integration (data not shown). In the presence of 9 mg of stromal protein per ml, as much as 15% of the added pLHCP was integrated into thylakoids during a 30-min incubation. Neither wheat germ extract (not shown) nor rabbit reticulocyte lysate (Fig. 1*B*, lane 3) could replace SE for pLHCP integration.

The above conclusions were made based on protease resistance of integrated (p)LHCP. It has been shown that membrane-integrated (p)LHCP is largely resistant to protease digestion, yielding a characteristic degradation product (LHCP-DP) upon treatment with proteases (18, 27, 28). Using resistance to alkali extraction as a criterion for integration, Yalovsky *et al.* (17) concluded that SE was not required for thylakoid integration of denatured/unfolded pLHCP. However, comparison of these two treatments showed that alkali extraction was not sufficiently rigorous to differentiate integrated from surface-bound pLHCP (compare Fig. 1 *A* and *B*). NaOH-resistant pLHCP was obtained with or without SE, with or without ATP (Fig. 1*A*). Alkali extraction was also not effective in removing pLHCP bound to the surface of intact chloroplasts (Fig. 1*A*). Similar conclusions have recently been reached by two other research groups even with *in vitro*-synthesized pLHCP (30, 31).

Hsp70 Is Not the Stromal Factor Required for pLHCP Integration. Yalovsky *et al.* (17) reported that urea-denatured pLHCP lost integration competence upon dialysis unless SE or purified Hsp70 was present during dialysis. We also observed that inclusion of Hsp70 during dialysis of urea-denatured pLHCP led to increased alkali-resistant pLHCP (Fig. 2*A*). However, integration of pLHCP as judged by protease resistance only occurred when SE was present during dialysis (Fig. 2*B*). We suggest that dialysis with Hsp70 increases association of pLHCP with the thylakoids because Hsp70 maintains pLHCP in solution throughout dialysis. In our experiment, most of the buffer-dialyzed pLHCP was adsorbed to the dialysis apparatus.

Payan and Cline (15) have previously correlated the integration-promoting activity of SE with its ability to convert pLHCP into a 120-kDa soluble complex. They showed that the plastid Hsp60 is not a component of the complex but could not determine whether the plastid Hsp70 was a component. To assess whether Hsp70 was part of the 120-kDa pLHCP soluble complex, we prepared an antibody to the plastid Hsp70 and used this antibody in immunoprecipitation analyses. Such analyses showed that the pLHCP soluble complex was not removed by treatment with anti-Hsp70 antibody (Fig. 3, lane 4). In contrast, all of the complex was removed by treatment with anti-LHCP antibody (Fig. 3, lane 2).

The possible involvement of Hsp70 in integration was further assessed by assaying pLHCP integration with Hsp70-depleted SE obtained by immunoaffinity chromatography on an anti-Hsp70 IgG protein A-Sepharose column. Control SE was treated identically with either a preimmune or a BSA mock-treated column. Depletion of Hsp70 was verified by Coomassie blue staining (Fig. 4*A*) and by immunoblotting with antibody to plastid Hsp70 or antibody to *E. coli* DnaK (Fig. 4*B*). By using a dilution calibration curve for immunoblotting with the antibody to plastid Hsp70, we determined that the depletion treatment removed >95% of the stromal Hsp70. Yet, the Hsp70-depleted SE was as competent as control SE for integrating pLHCP into thylakoids (Fig. 4*D*) or converting pLHCP into the 120-kDa soluble complex (Fig. 4*C*). Marshall *et al.* (32) reported that SE contains a second Hsp70. This second Hsp70 is present in very low amounts in SE and, although difficult to visualize by Coomassie staining, can be monitored by immunoblotting with an antibody against tomato cytosolic Hsp70 (19). With a similar dilution calibration curve, we determined that 50–60% of the minor Hsp70 was removed by our depletion treatment (data not shown). In the experiments with depleted SE, the stromal component was the limiting factor for integration (Fig. 5). The identical response of pLHCP integration to different amounts of control or Hsp70-depleted SE demonstrates that Hsp70 is not the stromal factor previously described.

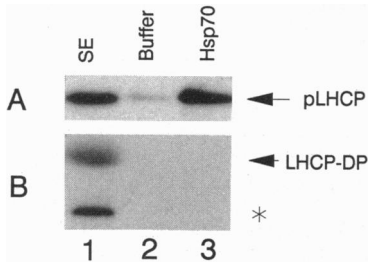

FIG. 2. Hsp70 is not able to support integration of purified pLHCP into isolated thylakoids. *E. coli*-made pLHCP was solubilized in 8 M urea/8 mM DTT for 4 hr at room temperature and then dialyzed to remove the urea before assay for integration. Dialysis was initiated by mixing 10 μl (≈6 μg) of urea-solubilized pLHCP with 400 μl of buffer A containing either 5 mg of stromal protein (lane 1), no addition (lane 2), or 100 μg of ATP-agarose affinity-purified stromal Hsp70 (lane 3). Dialysis was conducted at 4°C for 30 min against buffer A on no. 3 (molecular mass cutoff, 3500 Da) Spectra/por dialysis membrane (Spectrum Medical Industries). At the end of dialysis, 200 μl of the dialysis mixture was mixed with washed thylakoids and assayed for integration in the presence of 10 mM ATP. Recovered thylakoids were treated either by alkali extraction (*A*) or with thermolysin (*B*). LHCP-DP and marker *, see legend to Fig. 1.

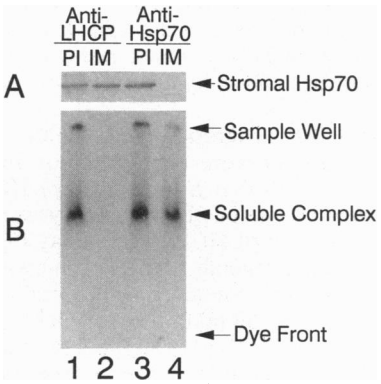

FIG. 3. Hsp70 is not part of the pLHCP soluble complex. ^{3}H-labeled pLHCP (translated *in vitro*) was mixed with SE and incubated for 15 min at 25°C in the presence of 5 mM ATP. The mixture was then subjected to immunoprecipitation analyses either with preimmune (PI) serum (lanes 1 and 3) or with immune (IM) serum (lanes 2 and 4). After removal of the pellet by centrifugation, the supernatant was analyzed for the presence of Hsp70 by immunoblotting (*A*) or for the presence of the soluble pLHCP complex by native gel electrophoresis (*B*).

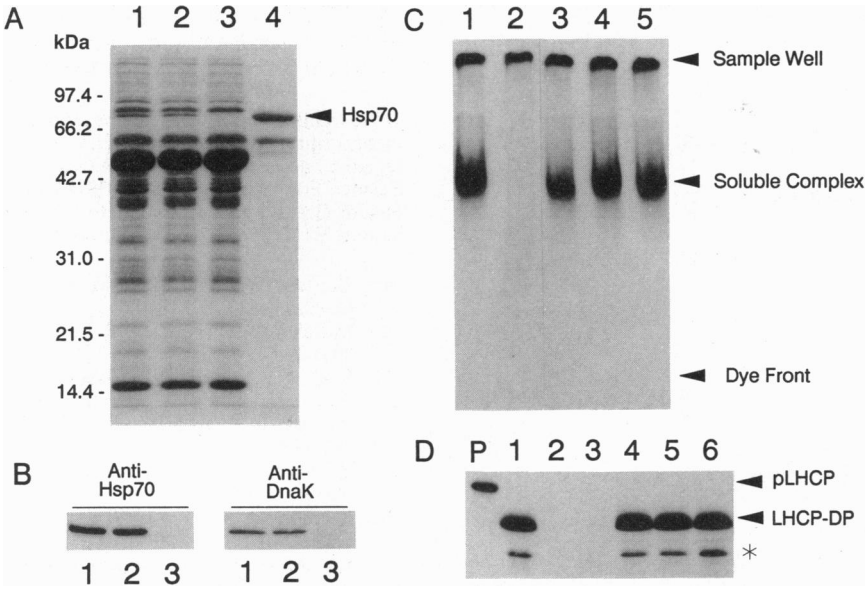

FIG. 4. Hsp70-depleted SE supports pLHCP integration equally well as does Hsp70-containing SE. Hsp70-depleted SE was prepared as described in the text. (*A*) Coomassie blue staining of stromal protein passed through a BSA mock column (lane 1), passed through a preimmune IgG column (lane 2), or passed through an anti-Hsp70 IgG column (lane 3). Lane 4, ATP-agarose affinity-purified stromal Hsp70 (shows the enrichment of Hsp70 as well as the presence of Hsp60). (*B*) Immunoblotting of stromal protein untreated (lane 1), passed through a BSA mock column (lane 2), or passed through an anti-Hsp70 IgG column (lane 3). (*C*) Complex formation with SE (lane 1), without SE (lane 2), or with SE passed through a BSA mock column (lane 3), passed through a preimmune IgG column (lane 4), or passed through an anti-Hsp70 IgG column (lane 5). (*D*) Integration with 1× SE (lane 1), without SE (lane 2), with purified stromal Hsp70 (lane 3), or with 1.5× SE passed through a BSA mock column (lane 4), passed through a preimmune IgG column (lane 5), or passed through an anti-Hsp70 IgG column (lane 6). Lane P, *in vitro*-translated pLHCP. LHCP-DP and marker *, see legend to Fig. 1.

Purified pLHCP, Diluted out of Urea, Is Stable as a Substrate for Import and Integration. The notion that unfolded pLHCP is sufficient for integration and that Hsp70 is the stromal factor presupposes that pLHCP rapidly folds into an integration-incompetent conformation. We examined this by diluting urea-denatured pLHCP to a low urea concentration (≈0.2 M) and then assaying aliquots for import and integration at various times after dilution. The amount of import or integration for each time point is plotted in Fig. 6 as a percentage of the no-preincubation control. The amount of import or integration was about 70% of the no-preincubation control when diluted pLHCP was preincubated for 30 min at 0°C and around 50% of the no-preincubation control when preincubated for 30 min at 25°C. Thus, although a time- and temperature-dependent decline in import and integration competence occurred, it was not rapid.

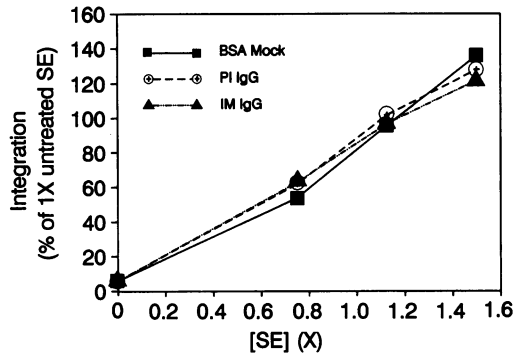

FIG. 5. Stromal protein concentration-dependent integration of pLHCP is independent of the presence of Hsp70. Quantification of pLHCP integration (see legend to Fig. 4 for details) with different concentrations (based on protein assays) of SE that had been depleted of Hsp70 with an anti-Hsp70 IgG column (IM IgG) or treated identically with a preimmune IgG column (PI IgG) or with a BSA mock column (BSA Mock). The protein concentration of 1× SE was 2.45 mg/ml (0.82 mg/ml final concentration in assay). Data are expressed as a percentage of the integration obtained with 1× untreated SE (1000 dpm/μg of Chl).

We also measured the amount of pLHCP present in each aliquot and found similar declines with time (data not shown). Presumably, pLHCP in 0.2 M urea is prone to adsorption to surfaces such as the plastic tubes and pipet tips used in this experiment. This further supports our earlier assertion (15) that measurable loss of pLHCP integration competence results from nonproductive reactions that lead to insolubility—e.g., aggregation and adsorption—rather than from folding.

DISCUSSION

Our data clearly show that whereas urea-denatured pLHCP is sufficient for import into intact chloroplasts (Fig. 1), integration into thylakoids requires a stromal factor that cannot be bypassed by unfolding (Fig. 1). The apparent integration reported by Yalovsky *et al.* (17) in the absence of SE can be entirely explained as unproductively associated pLHCP that was not removed by alkali extraction. Alkali extraction was originally designed to differentiate integral from peripheral membrane proteins in their native forms (33). Resistance to alkali extraction has traditionally been interpreted as evidence for protein–membrane interaction. However, caution must be taken when such a criterion is used to assess the behavior of denatured or nonnative proteins such as those used in transport studies. In the case of pLHCP integration, the current evidence indicates that the association of pLHCP with thylakoids in the absence of SE is not physiologically relevant.

Our experiments have also demonstrated that Hsp70 is not the stromal factor (Figs. 2, 4, and 5). We cannot rule out a role for Hsp70 in pLHCP integration because of the technical difficulty of removing all Hsp70 proteins from assay mixtures. In the experiment shown in Figs. 4 and 5, Hsp70 proteins (verified by immunoblotting) were associated with the washed thylakoids used in the assays and were present in the wheat germ extract used for preparing the pLHCP. It was

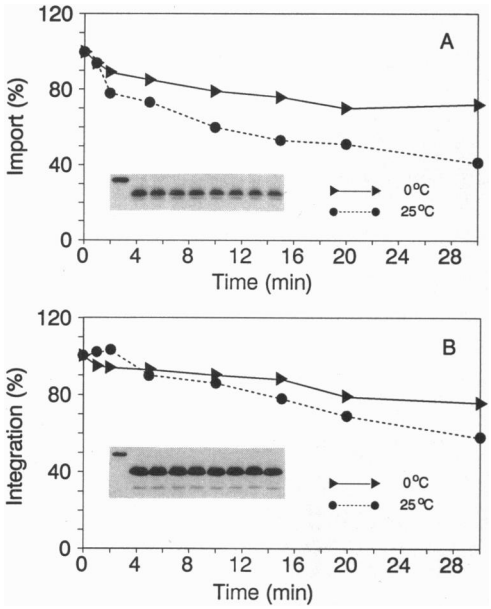

FIG. 6. Urea-denatured pLHCP remains competent for import and integration during incubation under renaturing conditions. *E. coli*-made pLHCP was denatured in 8 M urea/8 mM DTT at room temperature for 4 hr and then diluted to ≈0.2 M urea into import buffer plus 2 mM DTT. At various times after the dilution, pLHCP was assayed (at a calculated concentration of ≈0.2 μM) either for import into chloroplasts (*A*) or for integration into thylakoids in chloroplast lysates (*B*). Preincubation of pLHCP in buffer was performed at 0°C or at 25°C. Assays for import and integration were carried out in light (≈70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in the presence of 10 mM added ATP. The absolute number of pLHCP imported or integrated was, respectively, ≈5.5×10^4 or ≈5.7×10^4 molecules per chloroplast or chloroplast equivalent for the no-preincubation controls. About 14% of the added pLHCP was imported or integrated. (*Inset* in *A*) Fluorogram of import with pLHCP preincubated at 0°C. (*Inset* in *B*) Fluorogram of integration with pLHCP preincubated at 0°C.

necessary to use *in vitro*-translated pLHCP in the depletion experiments because it is not technically feasible to prepare Hsp70-depleted SE sufficiently concentrated for detectable integration of *E. coli*-produced pLHCP, owing to its much lower specific radioactivity. Nevertheless, our results show that plastid Hsp70 is not the stromal factor previously described. First, no integration occurs in the absence of SE even though Hsp70s are present on thylakoids and in the translation mixture (Fig. 4). Second, since the stromal factor is limiting in the integration reaction, its removal in total or in part would be reflected in a decrease in integration. As shown in Fig. 5, there is no difference in the integration-promoting activity between control and Hsp70-depleted SE.

It has been shown that premature folding can prevent membrane transport of a variety of authentic and chimeric preproteins (34–37). However, folding does not appear to be a problem for pLHCP import and integration. The apparent decrease in import and integration competence (Fig. 6) can be explained by the time-dependent loss of pLHCP from solution. This is not surprising as a stably folded pLHCP is only expected upon its interaction with the lipid bilayer. A molecular chaperone would help to prevent loss of pLHCP from solution. Indeed, our previous analyses demonstrated a chaperoning function for the stromal factor (15). However, such a role is not essential as demonstrated by the experiment in Fig. 6, where pLHCP lost only ≈50% of its initial competence during an incubation that mimicked the assay conditions for integration. The fact that the stromal factor is absolutely essential (Figs. 1 and 2) supports the proposal that the stromal factor has an additional function, most probably in targeting pLHCP to the membrane or possibly even in the mechanism by which pLHCP folds into the lipid bilayer.

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