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Multiple pathways for protein transport into or across the thylakoid membrane

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Many thylakoid proteins are cytosolically synthesized and have to cross the two chloroplast envelope membranes as well as the thylakoid membrane *en route* to their functional locations. In order to investigate the localization pathways of these proteins, we over-expressed precursor proteins in *Escherichia coli* and used them in competition studies. Competition was conducted for import into the chloroplast and for transport into or across isolated thylakoids. We also developed a novel *in organello* method whereby competition for thylakoid transport occurred within intact chloroplasts. Import of all precursors into chloroplasts was similarly inhibited by saturating concentrations of the precursor to the OE23 protein. In contrast, competition for thylakoid transport revealed three distinct precursor specificity groups. Lumen-resident proteins OE23 and OE17 constitute one group, lumenal proteins plastocyanin and OE33 a second, and the membrane protein LHCP a third. The specificity determined by competition correlates with previously determined protein-specific energy requirements for thylakoid transport. Taken together, these results suggest that thylakoid precursor proteins are imported into chloroplasts on a common import apparatus, whereupon they enter one of several precursor-specific thylakoid transport pathways.

Key words: chloroplast/oxygen-evolving complex/protein import/signal peptide/translocation

Introduction

Thylakoids are a closed internal membrane system within chloroplasts, separated from the delimiting outer and inner chloroplast envelope membranes by the aqueous matrix called the stroma. Yet many thylakoid proteins are encoded in the nucleus and synthesized in the cytosol. These proteins must traverse up to three membranes and three aqueous compartments *en route* from their site of synthesis to their functional locations [see de Boer and Weisbeek (1991) for review]. The localization pathway consists of two experimentally separable membrane translocation events: import of precursors across the envelope membranes into the stroma, and translocation of stromal intermediates into or across the thylakoid bilayer (Cline, 1986; Smeekens *et al.*, 1986; James *et al.*, 1989; Reed *et al.*, 1990).

Import across the envelope is governed by stromal targeting domains of amino-terminal transit peptides that are present on precursor proteins. Stromal targeting domains are removed by a protease located in the stroma [see de Boer and Weisbeek (1991) for review]. Import across the envelope is thought to be mediated by a common translocation mechanism because of the singular requirement for ATP as an energy source (Theg *et al.*, 1989, and references within) and because purified precursor proteins and synthetic transit peptides compete for the import of other precursor proteins (Perry *et al.*, 1991; Schnell *et al.*, 1991; Theg and Geske, 1992; Oblong and Lamppa, 1992).

Transport into or across the thylakoid membrane is governed by additional topogenic sequences. Transport of thylakoid lumen-resident proteins such as plastocyanin (PC) and OE33, OE23 and OE17 (the 33, 23 and 17 kDa subunits of the oxygen-evolving complex, respectively) is directed by 'lumen-targeting' domains of their transit peptides. Lumen-targeting domains share several features in common with bacterial signal peptides: a hydrophobic core region of ~15 residues, a positively charged N-flanking region and an A-X-A cleavage site (von Heijne *et al.*, 1989). Lumen-targeting domains are removed by a protease located in the thylakoid lumen (Halpin *et al.*, 1989). As a result of the bipartite nature of lumenal protein transit peptides, the stromal forms of lumenal proteins are intermediate in size between the full precursor and the mature protein. Integration of membrane proteins such as the light-harvesting chlorophyll a/b protein of photosystem 2 (LHCP) is directed by targeting signals within the mature protein sequence (Lamppa, 1988; Viitanen *et al.*, 1988). The exact location of these signals in LHCP has not been determined, but appears to lie within one or more of the three hydrophobic transmembrane segments (Auchincloss *et al.*, 1992).

Investigations of thylakoid protein translocation have been facilitated by the development of assays for transport into or across isolated thylakoid membranes (Cline, 1986; Kirwin *et al.*, 1989; Bauerle and Keegstra, 1991; Mould and Robinson, 1991; Cline *et al.*, 1992). Most studies with these reconstituted assays have used the full precursors produced by *in vitro* translation as substrates. In the case of OE33, OE23 and LHCP, forms lacking the stromal-targeting domains have been prepared and are also effective substrates (Viitanen *et al.*, 1988; Mould *et al.*, 1991; see below). Analyses of the energy requirements for thylakoid transport have shown them to be protein specific. Integration of LHCP and transport of OE33 and PC absolutely require ATP (Kirwin *et al.*, 1989; Bauerle and Keegstra, 1991; Cline *et al.*, 1992). LHCP integration and OE33 transport are further stimulated by a *trans*-thylakoid ΔpH (Mould and Robinson, 1991; Cline *et al.*, 1992). Conversely, transport of OE23 and OE17 requires only the thylakoidal ΔpH (Cline *et al.*, 1992).

If the varied energy requirements for thylakoid protein transport reflect distinct machinery, we would expect that such machinery would display precursor specificity. Here we have explored this possibility by conducting competition

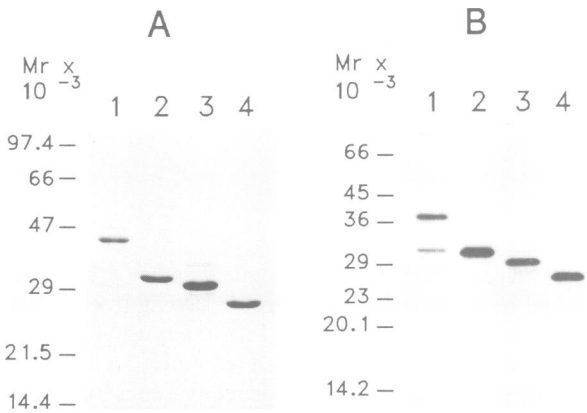

Fig. 1. Precursor proteins over-expressed in *E.coli*. The coding sequences for pOE33, pLHCP, pOE23 and iOE23 from pea were cloned into pET-based expression plasmids and introduced into BL21 (λDE3) (Materials and methods). Over-expression was induced with 1 mM IPTG and inclusion bodies containing the respective proteins were purified as described in Materials and methods. (**A**) Isolated proteins (1 μg) were subjected to SDS−PAGE and stained with Coomassie blue. (**B**) Proteins produced in cultures containing [^{3}H]leucine were subjected to SDS−PAGE and fluorography at 20 000 d.p.m./lane. Lane 1, pOE33; lane 2, pLHCP; lane 3, pOE23; lane 4, iOE23.

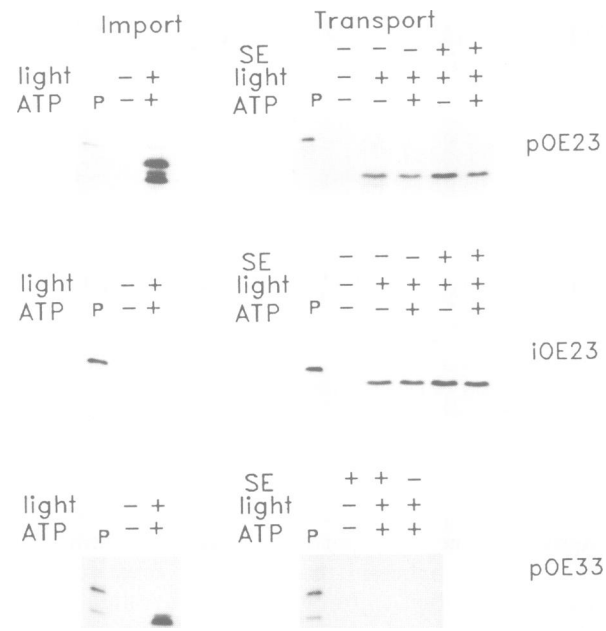

Fig. 2. Import of *E.coli*-produced precursors into intact chloroplast and transport across isolated thylakoid membranes. ^{3}H-labeled, *E.coli*-produced pOE33, pOE23 and iOE23 were dissolved in 8 M urea, 8 mM DTT and assayed for import into intact chloroplasts or transport into isolated thylakoids. Import assays (150 μl) were conducted with 3 μl of precursor in the dark or in the presence of 5 mM Mg-ATP and 70 μE m^{-2} s^{-1} white light for 10 min as shown and described in Materials and methods. Assays for translocation into isolated thylakoids (150 μl) received thylakoids and 3 μl precursor proteins, and were conducted for 30 min with or without 5 mM ATP, a stoichiometric quantity of stromal extract (SE), and light as shown above the panels and described in Materials and methods. The urea-denatured precursors were diluted directly into reaction mixtures. An SDS−PAGE fluorogram is shown in the figure. Gel lanes contain ~20% of the chloroplasts or thylakoids recovered from each assay and 2% of the precursor (lanes P) added to each assay.

experiments with precursor proteins obtained by over-expression in *Escherichia coli*. Our studies demonstrate that it is possible to saturate thylakoid transport with elevated concentrations of precursors and further show that there are precursor-specific components of the transport machinery that operate at the level of thylakoid binding and/or translocation.

Results

Production of purified precursor proteins

Chemical quantities of precursor proteins were obtained by over-expression in *E.coli*. The coding sequences for the full-length precursors pOE23, pOE33 and pLHCP, as well as the intermediate precursor iOE23, were cloned into pET plasmids (Materials and methods). iOE23 is the stromal form of OE23 that results from proteolytic removal of the stromal targeting peptide (Bassham *et al.*, 1991). Over-expressed precursors were sequestered into inclusion bodies. Isolation of inclusion bodies yielded precursor proteins ~90% pure by Coomassie staining (Figure 1A). Tritium-labeled precursors with similar radiochemical and chemical purity were obtained by including [^{3}H]leucine in the culture medium during induction (Figure 1B). Expression of pOE33 was less efficient than the other proteins and more variable. Large-scale preparations were required to obtain significant amounts of unlabeled pOE33; production of only small quantities of radiolabeled pOE33 was feasible. The pOE33 obtained was frequently contaminated with small amounts of a polypeptide with the same M_r as the mature size of OE33.

Saturation of thylakoid transport/integration with precursors produced in E.coli

Escherichia coli-produced pOE23 and iOE23, either added directly from 8 M urea (Figure 2) or prediluted to a lower urea concentration (~1 M or less), were competent for thylakoid transport. Transport characteristics were identical to those obtained with *in vitro* translated pOE23 (Cline *et al.*, 1992). Transport required energy in the form of a trans-thylakoid ΔpH. In Figure 2, the ΔpH was provided by conducting assays in the light. Transport was not increased by including ATP or stromal proteins in the assay (Figure 2). Residual urea was not required to maintain transport competence; iOE23 from which urea was completely removed by gel filtration was also transported without stroma (data not shown). pOE23 transport exhibited saturation kinetics (Figure 3A) with a K_m of ~0.19 μM and V_{max} of 700 molecules/chloroplast equivalent/min. This K_m is in a physiologically relevant range, i.e. half-maximal transport velocity would occur with several thousand molecules of stromal iOE23 per chloroplast. Such a concentration corresponds to estimates of the amount of protein translocation during chloroplast development (Pfisterer *et al.*, 1982). The above results demonstrate that elevated concentrations of pOE23 saturate a membrane component of the translocation apparatus.

Escherichia coli-produced pLHCP, either added directly from 8 M urea or pre-diluted to lower urea concentrations, was competent for thylakoid integration. Integration characteristics were identical to those previously described for *in vitro* translated pLHCP. Integration only occurred when stromal proteins (at least 2.5 mg/ml) and ATP were present during the reaction (Yuan *et al.*, 1993). Figure 3B

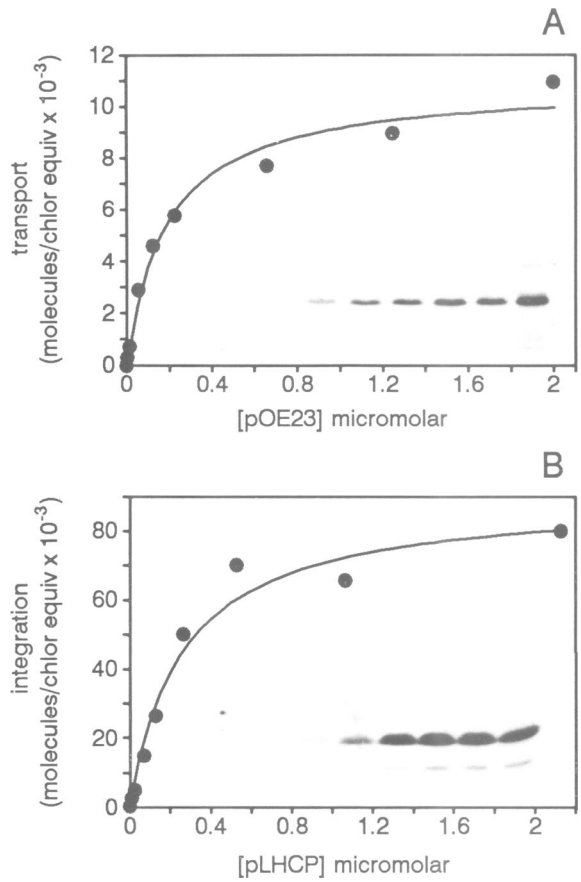

Fig. 3. Concentration dependence of pOE23 transport or pLHCP integration into isolated thylakoids. ^{3}H-labeled, *E.coli*-produced pOE23 and pLHCP were dissolved in 8 M urea, 8 mM DTT, pre-diluted with cold import buffer, 3 mM DTT, to 1.2 M urea (pOE23) or 0.4 M urea (pLHCP), and then immediately assayed for transport and integration, respectively. (**A**) pOE23 transport into isolated thylakoids. Each assay (150 μl) contained 50 μg chlorophyll of washed thylakoids, import buffer, 10 mM $MgCl_2$, 0.4 M urea, 1 mM DTT and pOE23. Reactions were conducted in the light for 15 min as described in Materials and methods. (**B**) pLHCP integration into isolated thylakoids. Each assay (350 μl) contained 100 μg chlorophyll of thylakoids, stromal extract equivalent to that present in 400 μg chlorophyll of chloroplasts, 34 mM HEPES/KOH (pH 8), 0.23 M sorbitol, 4 mM $MgCl_2$, 9 mM Mg-ATP, 0.22 M urea, 1 mM DTT and pLHCP. Reactions were conducted for 30 min in the light as described in Materials and methods. Substrate amounts added to each assay were determined by scintillation counting. The amount of transport or integration was determined by scintillation counting of bands extracted from the gel (Materials and methods) and is reported as molecules transported or integrated/chloroplast equivalent. The data points were best-fit to hyperbolic curves. Photographs of the fluorograms are inset in the figure.

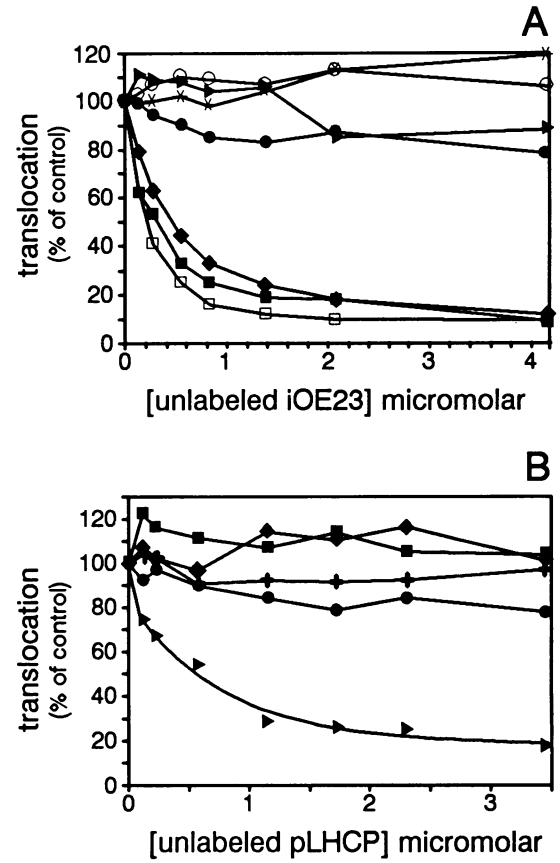

Fig. 4. iOE23 competes only for pOE23 and pOE17 transport into isolated thylakoids; pLHCP competes only for pLHCP integration into thylakoids. Translocation of radiolabeled precursor proteins was conducted with isolated thylakoids in the presence of increasing concentrations of either unlabeled iOE23 or pLHCP. (**A**) Unlabeled *E.coli*-produced iOE23 was dissolved in 8 M urea, 8 mM DTT, prediluted to 1.2 M urea with cold import buffer, 6 mM DTT, and then added to assay mixtures. Each assay (150 μl) contained 5 mM Mg-ATP, import buffer, 10 mM $MgCl_2$, 0.2 M urea, 1 mM DTT, 50 μg chlorophyll of chloroplast lysate, unlabeled iOE23 and radiolabeled precursor proteins produced by *in vitro* translation (Materials and methods). (**B**) Unlabeled *E.coli*-produced pLHCP was dissolved in 8 M urea, 8 mM DTT and then directly diluted into 15 vols of a stromal extract equivalent to 5.7 mg chlorophyll/ml. Varying unlabeled pLHCP concentrations were prepared by diluting this stock solution with stromal extract containing 0.5 M urea. Each assay (150 μl) contained 50 μg chlorophyll of lysate, stromal extract equivalent to 250 μg chlorophyll, 20 mM HEPES/KOH (pH 8), 70 mM sorbitol, 8 mM $MgCl_2$, 5 mM Mg-ATP, 0.3 M urea, unlabeled pLHCP and radiolabeled precursor proteins produced by *in vitro* translation. All reactions were conducted in the light for 15 min, and were quantified as described in Materials and methods and Figure 3. The radiolabeled precursor proteins used in the above assays were: *-*, pOE33; +-+, iOE33; ■-■, pOE23; □-□, iOE23; ◆-◆, pOE17; ▶-▶, pLHCP; ●-●, pPC from pea; ○-○, pPC from *Arabidopsis*.

shows the concentration dependence of pLHCP integration in the presence of stromal proteins equivalent to 4-fold the stoichiometry present in chloroplasts (~10 mg/ml). pLHCP integration exhibited saturation kinetics with a K_m of ~0.38 μM and V_{max} of 3000 molecules/chloroplast equivalent/min.

We observed no significant transport of *E.coli*-produced pOE33 into isolated thylakoids (Figure 2). The reason for this is unclear, but it may relate to the inefficiency of pOE33 as a thylakoid transport substrate. Even *in vitro* translated pOE33, with a substantially higher specific radioactivity, performs relatively poorly in our thylakoid transport assay.

Competition for transport with saturating amounts of substrate

Thylakoid transport of radiolabeled *in vitro* translated precursor proteins was conducted in the presence of increasing concentrations of unlabeled *E.coli*-produced precursor proteins (Figure 4). Unlabeled p- or iOE23 inhibited pOE17 transport, but did not affect pOE33 transport, pPC transport or pLHCP integration (Figure 4A). Inhibition occurred at the concentrations shown above to saturate the translocation apparatus, and inhibition curves for transport of radiolabeled iOE23, pOE23 and pOE17 were

virtually identical. In this particular experiment, iOE23 was used as the unlabeled competitor; similar results were obtained with pOE23. Stromal proteins were present in the pOE23 and pOE17 assays shown in Figure 4A. However, similar inhibition curves were obtained in the absence of stroma (data not shown). Thus, inhibition of pOE17 transport resulted from i- or pOE23 substrate competition for a membrane component of the translocation apparatus.

Unlabeled pLHCP inhibited integration of radiolabeled pLHCP, but did not affect transport of the lumenal proteins (Figure 4B). Inhibition occurred at the concentrations shown above to saturate the integration reaction (Figure 3B). In preliminary experiments, transport of pOE33 was occasionally inhibited by elevated concentrations of pLHCP (40–50% inhibition with concentrations >1.5 μM pLHCP). However, as shown in Figure 4B, inhibition did not occur if iOE33 was used as the radiolabeled substrate. This indicates that the observed inhibition resulted from secondary effects related to the stromal targeting domain, possibly a requirement for proteolytic processing, rather than inhibition at the level of translocation.

These results indicate that OE23 and OE17 share at least one saturable component of the transport machinery, that LHCP uses a different saturable component, and that OE33 and PC do not use these components. Competition assays with isolated thylakoids could not be used to determine if PC and OE33 share common component(s) because of the inability of *E.coli*-produced pOE33 to be transported in this reconstituted assay and because we have not yet been able to over-express pPC in *E.coli*. An alternative approach to this question was made possible by our unexpected observation that thylakoid transport of OE23 and OE33 can be saturated within intact chloroplasts during an *in organello* import assay.

Saturation of OE23 and OE33 thylakoid transport can occur during import into intact chloroplasts

All of the full-length precursors described above were competent for import into intact chloroplasts (see Figure 2 for pOE33 and pOE23 and Yuan *et al.*, 1993 for pLHCP). Efficient import occurred without added cytosolic factors and was dependent upon the presence of ATP (Figure 2).

Import of pOE23 into chloroplasts exhibited Michaelis–Menten kinetics with a K_m of ~0.4 μM and a V_{max} of ~40 000 molecules/chloroplast/min, i.e. a total of ~400 000 molecules/chloroplast for the 10 min assay (Figure 5A). This K_m is somewhat higher than the value determined by Pilon *et al.* (1992) for import of purified preferredoxin. The difference may relate to different affinities of precursor proteins for the envelope translocation apparatus or the behavior of different precursors diluted from denaturant.

At precursor concentrations >0.5 μM, most of the imported OE23 accumulated as iOE23. Subfractionation of recovered chloroplasts indicated that the accumulated iOE23 was soluble. iOE23 was recovered predominantly in the 40 000 *g* × 30 min supernatant fraction of lysed chloroplasts and eluted from a Superose 6 gel filtration column (Pharmacia) with an M_r of ~20 000 (data not shown). The stromal volume of chloroplasts is ~15 μl/mg chlorophyll (Cline *et al.*, 1984). From this it can be calculated that iOE23 accumulated to ~25 μM, a concentration that far exceeds the amount necessary to saturate the OE23 thylakoid transport machinery (Figure 3A). Thus, import of pOE23 into the chloroplast can concentrate the physiological substrate for thylakoid transport to supersaturating levels.

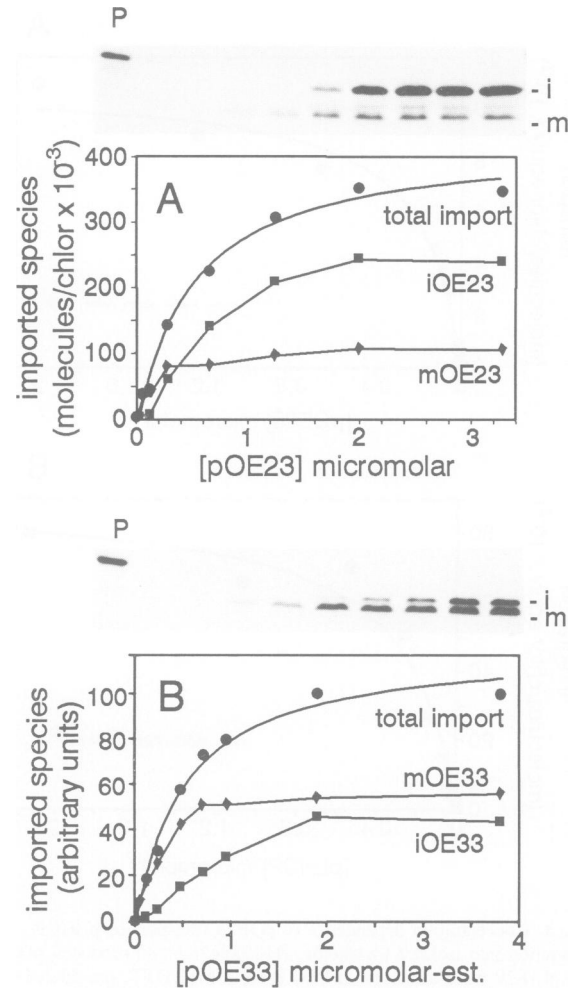

Fig. 5. Concentration dependence of pOE23 and pOE33 import into chloroplasts. (**A**) Tritium-labeled *E.coli*-produced pOE23 was prepared as described in Figure 3. Assays (150 μl) contained intact chloroplasts equivalent to 50 μg chlorophyll, 10 mM Mg-ATP, 1.3 mM DTT and 0.3 M urea. Assays were conducted for 10 min in the light and quantified as described in Materials and methods. Results are reported as molecules imported/chloroplast for the 10 min incubation. (**B**) *Escherichia coli*-produced pOE33 (200 μg) was dissolved in 31 μl 8 M urea, 8 mM DTT for 4 h, then diluted with 100 μl import buffer, 6 mM DTT and 250 μl of an adjusted pOE33 translation mixture (Materials and methods). pOE33 substrate stock solutions were prepared by mixing aliquots of the above mixture with a solution containing import buffer, 0.66 M urea, 2 mM DTT and 33% mock translation reaction such that the final concentration of all components except the labeled and unlabeled pOE33 was the same in each assay mixture. The final concentration of urea was 0.33 M. Import into intact chloroplasts was conducted as described in (A). The displayed quantities of imported species were obtained by scintillation counting of proteins extracted from gel bands (Materials and methods) with an adjustment for the number of leucines/molecule of intermediate (i) and mature (m) species. The data points for total import were best-fit to hyperbolic curves. Photographs of the fluorograms are shown above plots of the data. Lanes P contain aliquots of the precursors used in these assays.

Increasing concentrations of pOE33 produced a similar profile for import into chloroplasts. In this case, the substrate consisted of a mixture of unlabeled *E.coli*-produced pOE33 and radiolabeled pOE33 prepared by *in vitro* translation. This was necessary because of the inability to produce large

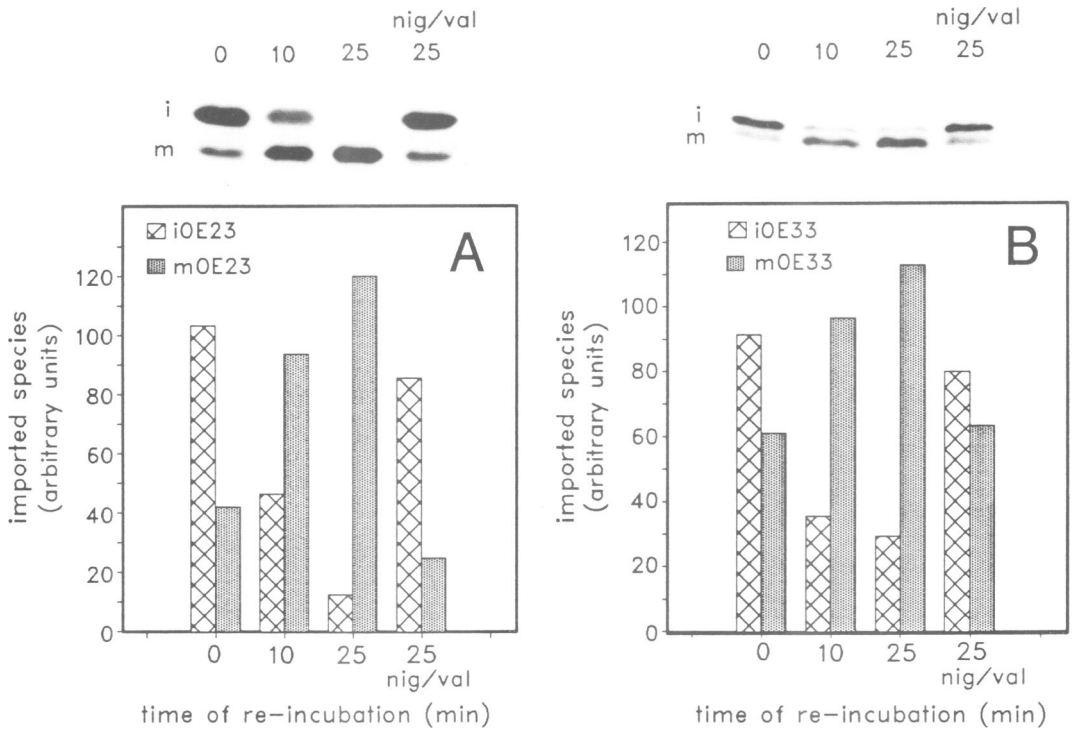

Fig. 6. Stromal iOE23 and iOE33 are on the transport and assembly pathway. Chloroplasts were pre-incubated with unlabeled pOE23 (0.8 μM) or pOE33 (0.95 μM) at 25°C for 5 min in the presence of 5 mM ATP in the light; radiolabeled precursors produced by *in vitro* translation were then added and the reaction continued for an additional 5 min. Unlabeled precursors were prepared by dissolving in 8 M urea, 8 mM DTT and then diluting with import buffer, 6 mM DTT, to 0.5 M urea. Final urea concentration in reaction mixtures was 0.08 M. After incubation, reaction mixtures were chilled on ice, the chloroplasts repurified on Percoll cushions and washed once with import buffer. Chloroplasts were then resuspended in import buffer containing 5 mM Mg-ATP to ~1 mg chlorophyll/ml and divided into four equal aliquots. One was immediately frozen, one incubated for 10 min at 25°C with illumination, one for 25 min, and one for 25 min in the presence of 0.5 μM nigericin and 1.0 μM valinomycin. Samples were mixed with an equal volume of SDS buffer and subjected to quantitative SDS–PAGE and fluorography. Equivalent amounts of chloroplasts were applied to each gel lane and the radiolabeled proteins quantified as described in Materials and methods and Figure 5. The intermediate (i) and mature (m) species are designated next to the fluorograms. Washed chloroplasts contained no detectable pOE23 or pOE33. (**A**) Chase of iOE23. (**B**) Chase of iOE33.

amounts of ^{3}H-labeled pOE33. Michaelis–Menten kinetics were observed for total protein import into the chloroplast (Figure 5B). At intermediate substrate concentrations, iOE33 accumulated within the plastid. iOE33 fractionated with the soluble chloroplast fraction (supernatant of a 40 000 *g* centrifugation) and eluted from a Superose 6 gel filtration column primarily as a monomeric species (data not shown).

Import of pLHCP displayed similar saturation kinetics, but there was no significant accumulation of stromal LHCP (data not shown). This can be explained by a relatively lower V_{max} for pLHCP import, ~25% of that observed for pOE23 import, and a relatively higher V_{max} for pLHCP integration.

Accumulated intermediates remain on-pathway

The iOE23 and iOE33 accumulated during import assays were shown to be on the transport pathway by a chase experiment. This was accomplished by repurifying chloroplasts from the reaction mixture after a substantial quantity of intermediates had accumulated and then re-incubating the chloroplasts in ATP-containing buffer (Figure 6). During re-incubation, iOE23 and iOE33 diminished in parallel with compensating increases in mOE23 and mOE33, respectively (Figure 6). Inclusion of ionophores, previously shown to inhibit thylakoid localization of these proteins (Cline *et al.*, 1992), prevented this maturation process. These results indicate that iOE23 and iOE33 that accumulate during import of large amounts of precursor proteins are pathway intermediates and are transport competent.

Thylakoid precursor proteins share at least one common component for transfer across the envelope membranes

The above results suggested that appropriate selection of precursor concentration would allow for competition either for import into the chloroplast or for transport of the imported proteins into the thylakoids. An import-saturating concentration of unlabeled pOE23 (3.5 μM) was used to compete for import of radiolabeled proteins into the chloroplast. Import of all the thylakoid precursor proteins tested (pOE33, pOE23, pOE17, pPC, pLHCP) was severely inhibited (Figure 7, lanes 2). Inhibition ranged from 75 to 95% depending on the precursor protein. Similar inhibition was observed for the precursor to the small subunit of Rubisco (pSS), a stromal protein precursor. Elevated concentrations of iOE23 served as a control for this experiment. iOE23 produced in *E.coli* was competent for transport into isolated thylakoids, but was not imported into intact chloroplasts because it lacks the stromal targeting domain of the transit peptide (Figure 2). As shown in Figure 7 (lanes 3), iOE23 had no significant effect on import

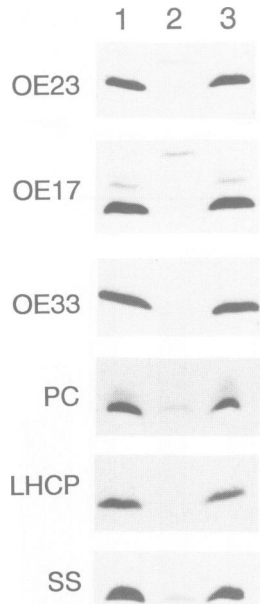

Fig. 7. pOE23 competes with thylakoid as well as stromal precursor proteins for import into intact chloroplasts. Import of radiolabeled precursors was conducted in the absence or presence of unlabeled pOE23 or unlabeled iOE23. Unlabeled precursors were prepared by dissolving inclusion bodies in 8 M urea, 8 mM DTT, diluting to 0.4 M urea with import buffer, 6 mM DTT, and then added to reaction mixtures that contained chloroplasts and 5 mM Mg-ATP. Radiolabeled precursors prepared by *in vitro* translation were added and import reactions conducted in the light for 10 min. Chloroplasts were protease-treated and repurified prior to SDS−PAGE and fluorography. Final urea concentration in all assays was 0.2 M. Lane 1, no addition; lane 2, 3.5 μM unlabeled pOE23; lane 3, 4.0 μM unlabeled iOE23.

of the precursors tested. This indicates that competition resulted from a specific interaction with envelope machinery rather than from non-specific effects of non-native OE23 or from titration of a general chaperone protein. These results provide strong support for the notion that thylakoid proteins as well as stromal proteins use at least one common component of the machinery for import into the chloroplast. A similar conclusion was reached in other studies with fewer precursor proteins (Perry *et al.*, 1991; Schnell *et al.*, 1991; Theg and Geske, 1992; Oblong and Lamppa, 1992).

iOE23 competes for thylakoid transport of iOE17 in organello during an import assay; iOE33 competes for the thylakoid transport of iPC

Inclusion in import assays of unlabeled pOE23 or pOE33 at concentrations subsaturating for import, but saturating for thylakoid transport, allowed us to conduct thylakoid competition studies with intact chloroplasts (Figure 8). Import of radiolabeled precursors into the chloroplasts was only slightly diminished. However, certain combinations of unlabeled and labeled precursors led to accumulation of radiolabeled stromal intermediates. Import in the presence of unlabeled pOE23 resulted in accumulation of radiolabeled iOE23 and iOE17 (lanes T). These were recovered in the soluble chloroplast fraction (lanes S). Unlabeled pOE23 did not affect localization of imported OE33 and PC to the thylakoids (lanes M).

Import in the presence of unlabeled pOE33 resulted in the accumulation of iOE33 and of two intermediate-size PC species in the soluble chloroplast fraction (lanes S). The two

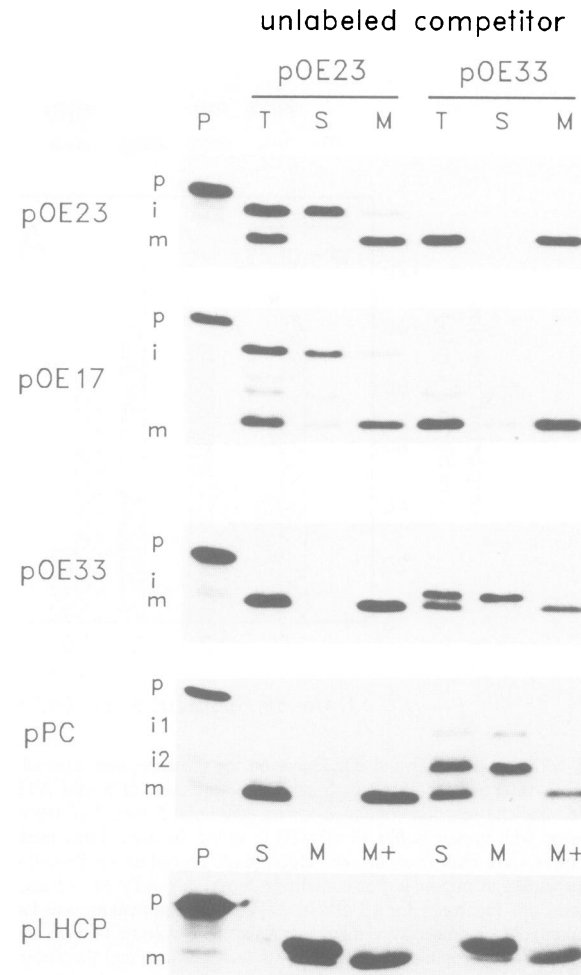

Fig. 8. iOE23 competes for thylakoid transport of iOE17; iOE33 competes for thylakoid transport of iPC *in organello* during import into intact chloroplasts. Import of radiolabeled pOE23, pOE17, pOE33, pPC and pLHCP prepared by *in vitro* translation was conducted in the presence of 0.8 μM unlabeled pOE23 or 0.95 μM unlabeled pOE33. Assays (600 μl) were conducted as described in Figure 6. Chloroplasts recovered from assays were post-treated with thermolysin, repurified and lysed with 50 μl of 10 mM HEPES/KOH (pH 8) for 5 min followed by addition of 50 μl of 2 × import buffer. Soluble and membrane fractions were separated by centrifugation at 3200 *g* for 10 min. Stoichiometric quantities of subfractions were applied to each gel lane. Membrane fractions recovered following pLHCP import were also treated with protease (Materials and methods). In the figure, the radiolabeled precursor used in each experiment is designated next to the panel, whereas the unlabeled competitor (pOE23 or pOE33) is designated above the panel. Lane designations are unfractionated chloroplasts (T), soluble (S), membrane (M) and protease-treated membrane (M+) fractions. Gel lanes contain ~10% of the chloroplasts or subfractions recovered from each assay and 1% of the radiolabeled precursor (lanes P) added to each assay. Precursor (p), intermediate (i) and mature (m) forms are indicated.

PC intermediates have been observed during time course analysis of import into intact chloroplasts (C.Li and K.Cline, unpublished). Unlabeled pOE33 had no effect on the localization of imported OE23 or OE17.

Imported LHCP was properly localized within the plastid in assays containing either unlabeled pOE23 or pOE33. For analysis of LHCP localization, the recovered chloroplasts were subfractionated into soluble (S) and membrane (M) fractions, and the membranes protease treated (M+) to determine the integration status (correctly integrated LHCP

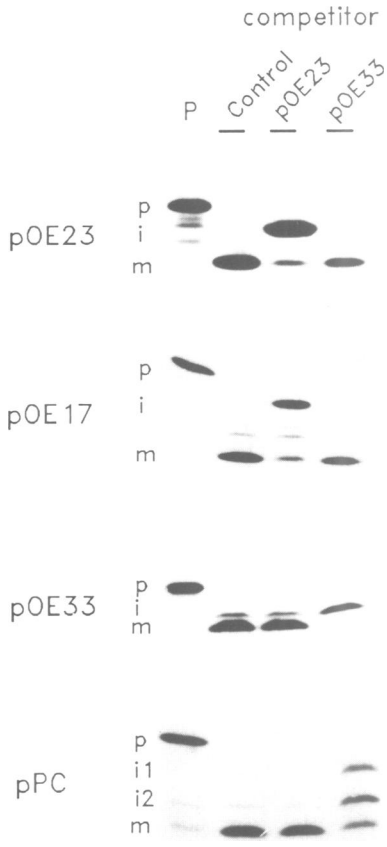

Fig. 9. Rapid stopping of *in organello* competition experiments reveals the extent of thylakoid transport inhibition. Import of radiolabeled pOE33, pOE23, pOE17 and pPC was conducted in the presence of unlabeled pOE23 (1.1 μM) and pOE33 (1.2 μM), as described in Figures 6 and 8. Reactions were stopped by addition of $HgCl_2$ to 3.3 mM and transfer to ice. Chloroplasts were then protease treated and repurified as described by Reed *et al.* (1990). A fluorogram of the SDS–polyacrylamide gel is shown. Gel lanes received ~20% of the chloroplasts recovered from each assay and 1% of the precursor added to each assay. Designations are as in Figure 8.

is characteristically partially resistant to protease). No significant differences could be detected between control (data not shown) and pOE23- or pOE33-containing assays. Thus, transport-saturating amounts of iOE23 or iOE33 did not interfere with LHCP integration.

Rapid termination of in organello competition assays reveals the extent of thylakoid transport inhibition

The relative amounts of intermediates that resulted from *in organello* competition experiments depended on the manner in which the reactions were terminated. We observed relatively less intermediate in assays that were terminated with the lengthy protease post-treatment and repurification procedures (Figure 8) than those simply pelleted and washed (Figure 6). This suggested that transport into thylakoids was occurring during the workup procedures. In order to determine the extent of *in organello* competition at the end of the incubation period, assays were rapidly stopped with 3.3 mM $HgCl_2$. This procedure has previously been shown to terminate both import into the plastid and all intra-plastid reactions (Reed *et al.*, 1990). Under these conditions, nearly all of the imported OE23 and OE17 accumulated as intermediates in the presence of unlabeled pOE23. Similarly, in the presence of unlabeled pOE33, virtually all of the imported OE33 and most of the imported PC accumulated as intermediates (Figure 9).

Discussion

Previous analysis showed that the energy requirements for protein transport into or across the thylakoid membrane are precursor protein specific (Cline *et al.*, 1992). Here we have used substrate competition studies to reveal three distinct classes of precursor-specific transport machinery: one for OE23 and OE17, one for OE33 and PC, and a third for LHCP. Evidence for substrate-saturable components includes the Michaelis–Menten kinetics exhibited by pOE23 transport and pLHCP integration into isolated thylakoids (Figure 3), as well as the apparent saturation of thylakoid transport by iOE23 and iOE33 *in organello* during import into intact chloroplasts (Figure 5). It is clear that a membrane component is titrated by elevated p- or iOE23 concentrations. Previous studies showed that transport of *in vitro* translated pOE23 or pOE17 occurred without added stroma (Cline *et al.*, 1992). Here we were able to demonstrate that purified p- or iOE23 are efficiently transported into washed thylakoids, even in the absence of denaturants (Figures 2 and 3). In the case of LHCP and OE33, it could not be definitively determined whether membrane components or soluble factors were titrated by elevated substrate concentrations. LHCP (Payan and Cline, 1991), as well as OE33 (J.Yuan and K.Cline, in preparation), require stromal components for thylakoid translocation. Nevertheless, the competition studies shown in Figures 4 and 8 demonstrate that the saturable components in these reactions are precursor specific.

The ability to saturate thylakoid transport *in organello* during import assays provided a novel and more physiologically relevant means to conduct competition experiments. A variety of investigations have shown that non-productive reactions, such as misfolding and/or aggregation, occur when proteins are diluted from denaturant (Mitraki and King, 1989, and references within). Although unlikely, the possibility of secondary effects in competition studies with isolated thylakoids could not be ruled out. *In organello* competition circumvents such problems because the envelope acts as a filter to selectively pass only those molecules that are translocation competent; it presents the thylakoid translocation machinery with the substrate forms likely to occur *in vivo*. The results in Figure 6 show that even though iOE23 and iOE33 accumulated to relatively high concentrations within the chloroplast, they remained transport competent.

The results obtained from chloroplast import assays are entirely consistent with those obtained from assays with isolated thylakoids. The fact that the V_{max} for pOE23 import into the plastid is ~40-fold higher than the V_{max} obtained for transport into isolated thylakoids predicts that thylakoid transport would be the slow step and explains the build-up of iOE23. Moreover, that iOE23 attained a stromal concentration many times the K_m for thylakoid transport predicts that substrate competition for thylakoid transport would occur under such conditions. In fact, all of the *in organello* competition results were internally consistent and consistent with competition studies using isolated thylakoids. This argues strongly that the accumulation of stromal inter-

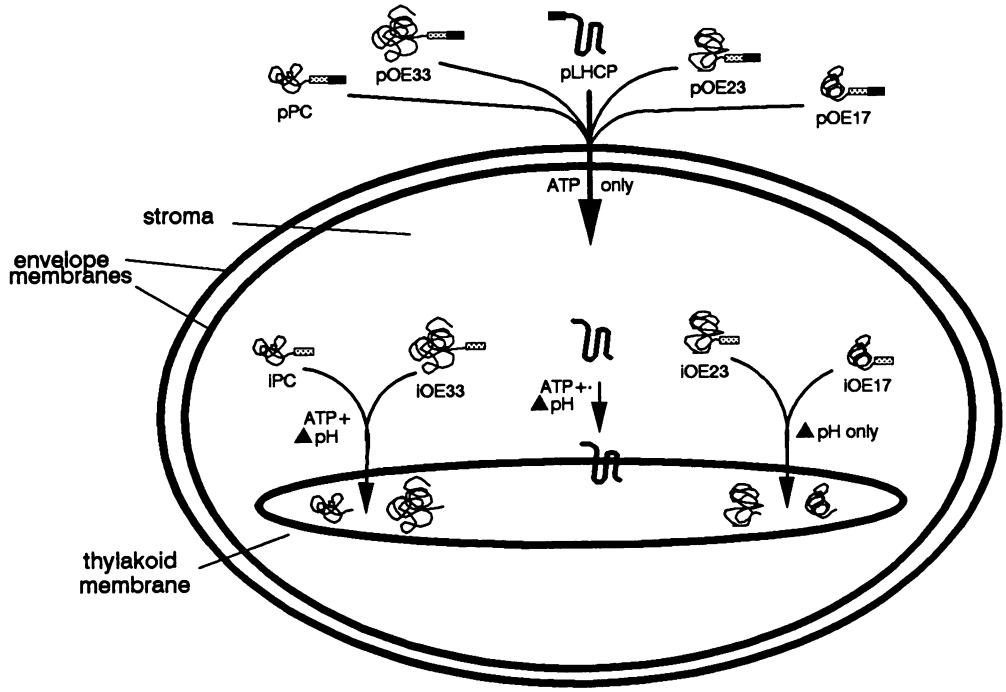

Fig. 10. Model for import and assembly pathways of nuclear-encoded thylakoid proteins.

mediates shown in Figures 8 and 9 resulted from substrate competition for the thylakoid transport machinery rather than from secondary effects or from interference with a general import/assembly component such as the cpn60 complex (Lubben *et al.*, 1989).

Our competition results are in agreement with the previously described energy requirements for thylakoid transport (Kirwin *et al.*, 1989; Bauerle and Keegstra, 1991; Mould and Robinson, 1991; Cline *et al.*, 1992). OE23 and OE17 transport requires a thylakoidal ΔpH alone. LHCP integration requires ATP and is stimulated by a ΔpH. OE33 and PC transport requires ATP. Although it is generally agreed that OE33 transport is stimulated by a proton gradient, several investigations have concluded that PC transport occurs independently of ΔpH because protonophores do not affect the localization of PC during import into intact chloroplasts (Theg *et al.*, 1989; Cline *et al.*, 1992). However, we have recently reconstituted PC transport across isolated thylakoids; our studies confirm the absolute requirement for ATP and also show a small but consistent enhancement by the proton gradient (J.Yuan and K.Cline, in preparation).

The studies reported here clearly demonstrate the presence of precursor-specific translocation components. Presumably, the specificity is mediated by sequences present in the lumen-targeting peptides and corresponding receptor-like components of the translocation apparatus. Homology analysis of lumen-targeting domains suggests the presence of specific elements. The lumen-targeting sequences of proteins from a wide range of plant species fall into two groups: one contains the known OE23 and OE17 targeting peptides; the other contains PC and OE33 peptides (M.McCaffery and K.Cline, unpublished results). One possible explanation for our results is that there are specific receptors, but that other components of the translocation machinery are common. Precedent for such a situation exists in other systems. For example, a common mitochondrial 'general insertion protein' is utilized by precursors that initially bind to different receptors (see Segui-Real *et al.*, 1992, for review). In this case, the common step was revealed by precursor competition studies (Pfaller *et al.*, 1988). On the other hand, the correlation of precursor specificity with the different energy requirements is a more persuasive argument that there are distinct translocation systems because studies with mitochondria and bacteria have shown energy requirements to be intimately related to the steps of translocation processes (Neupert *et al.*, 1990; Driessen, 1992). Additional support for this view comes from our recent observations that sodium azide is a potent inhibitor of thylakoid transport of OE33 and PC, but not of OE23 or OE17 transport or LHCP integration (J.Yuan and K.Cline, in preparation). Sodium azide is known to inhibit *E.coli* protein export by its action on SecA (Oliver *et al.*, 1990). A working model based on these considerations is presented in Figure 10. Future investigations will address the question of common components.

Hartl and Neupert (1990) have proposed a 'conservative sorting' hypothesis for the routing of certain nuclear-encoded chloroplast and mitochondrial proteins, in which precursors are imported into the matrix and then enter ancestral assembly pathways that derive from the endosymbiont. Our results are consistent with this model for thylakoid proteins. Conservative sorting predicts a general import translocator; our results describe an import apparatus with a relatively high V_{max} (Figure 5) that carries both stromal and thylakoid-destined proteins (Figure 7). Secondly, this model predicts that processes within the organelle would be more highly tailored to each protein as both undoubtedly co-evolved within the endosymbiotic progenitor. Our studies of thylakoids show protein-specific transport components and energy requirements, and substantially lower V_{max} values.

Yet, the apparent multiplicity of transport pathways for so few proteins examined is somewhat surprising. Conservative sorting suggests that intraorganellar transport

systems resemble those of modern day prokaryotes. Gram-negative bacteria utilize a general system for translocation of signal peptide-bearing proteins as well as some integral membrane proteins (Pugsley, 1993). It employs SecA and SecY/E proteins, and is powered by the combined action of ATP hydrolysis and protonmotive force. It remains to be determined if either PC/OE33 transport or LHCP integration is homologous to the SecA, SecE/Y system, but the pathway for OE23/OE17 transport is likely to be more distantly related because it functions without ATP. The evolutionary origin of the OE23/OE17 pathway is presently uncertain. Cyanobacteria contain PC and OE33, but OE23 and OE17 are found only in plants and algae. It is not currently known whether these proteins appeared after the endosymbiotic event or rather have been lost from cyanobacteria. Similarly, there is no known prokaryotic homologue of LHCP. Thus, the specificity observed here seems to correlate with evolutionary origins of the translocated proteins.

Materials and methods

Materials

All reagents, enzymes and standards were from commercial sources. Several *in vitro* expression plasmids for the precursors from pea were used in this study. Plasmids psAB80XD/4, pOE33, pOE23, pOE17 and pPC1, which harbor the coding sequences for pLHCP, pOE33, pOE23, pOE17 and pPC from pea, respectively, have been described previously (Cline *et al.*, 1992). Plasmid pSPPCara, an SP6 plasmid containing the gene for pPC from *Arabidopsis*, was provided by Dr Peter Weisbeek, University of Utrecht, The Netherlands (Bauerle and Keegstra, 1991). Plasmid pSMS64 contains the coding sequence for pSS of Rubisco (Anderson and Smith, 1986). Plasmid iOE33SEL is a pSELECT (Promega) plasmid containing the coding sequence for the OE33 intermediate (iOE33). The iOE33 coding sequence was amplified by polymerase chain reaction (PCR) using pOE33 as template. The forward primer, 5′-GGTGTAACCATGGCCTTTGGTTTGGAACA-CTATGG-3′, and reverse primer, 5′-GGAGTTTAGAGCTCAACAAA-CCATAACAAAGAAGC-3′, contained an in-frame *Nco*I site that makes up the initiation ATG and an *Sst*I restriction site, respectively. The forward primer was chosen to produce translation product starting with MAFGLEH..... This is in accordance with the published amino terminus of the OE33 stromal intermediate (Bassham *et al.*, 1991) with addition of the initiator methionine. The PCR product was digested with *Nco*I and *Sst*I, and ligated with a modified pSELECT by standard procedures (Crowe *et al.*, 1991).

Several plasmids for over-expression of proteins in *E.coli* were prepared for this study. The coding sequences for pLHCP, pOE23 and iOE23 were amplified by PCR using psAB80XD/4 and pOE23 as templates, respectively. Primers for amplification of pLHCP were 5′-GCAATATAATACCATA-TGGCCGCATCATCATCATC-3′ and 5′-CGTAGGTAGCTCGAGATA-ATCACTTGGATACGTATGGG-3′, containing an in-frame *Nde*I site that makes up the initiation ATG and a 3′ *Sst*I site. Primers for pOE23 amplification were 5′-GAACTCTGTATACCGTAGATGTGTTACAAA-GAACG-3′ and 5′-GCAACAGGATATCACCAAAGTTCTTGATTA-GGC-3′, containing in-frame *Nde*I and *Eco*RV sites, respectively. The forward primer for amplification of the iOE23 sequence was 5′-CCATA-TTGTTCATATGGCACAGAAACAAGATG-3′. This was chosen to produce a protein product starting with MAQKQDDVVD.... according to the published amino terminus of the OE23 stromal intermediate (Bassham *et al.*, 1991) with the addition of the initiator methionine. The reverse primer was that used for pOE23 amplification. The PCR products were digested with *Nde*I and *Sst*I (pLHCP) or *Nde*I and *Eco*RV (pOE23, iOE23), and ligated with appropriately digested pETH3c (McCarty *et al.*, 1991) plasmids to produce pETHPLHCP, pETHPOE23 and pETHIOE23, respectively. For pLHCP, the desired fragment was a partial restriction product. These plasmids were introduced into BL21 (λDE3)plysS. The expression clone for pOE33 was generously provided by Dr Steven Theg and was prepared by ligating a *Nco*I–*Bam*HI restriction fragment of pOE33 into a similarly digested pET 8c. This plasmid was introduced into BL21(λDE3).

Preparation of radiolabeled precursors by in vitro translation

Capped RNA for the various precursors was produced *in vitro* essentially as described by Cline (1988) using the following polymerases: T3 polymerase for pOE23, T7 polymerase for pOE33 and iOE23, and SP6 polymerase for pSS, pLHCP, pOE17 and pPC. The templates psAB80XD/4, pOE17, pOE23, pOE33, pPC1, pSPPCara, iOE33SEL, pSMS64 and pETHIOE23 were linearized with *Eco*RI, *Xba*I, *Hin*dIII, *Bam*HI, *Eco*RI, *Xba*I, *Eco*RI, *Eco*RI and *Eco*RV, respectively. RNA was translated in the presence of [^{3}H]leucine in a wheat germ system (Cline *et al.*, 1989). Translations were terminated by transfer to ice, dilution, and adjusting the mixture to import buffer [50 mM HEPES (pH 8), 0.33 M sorbitol] and 30 mM of unlabeled leucine.

Over-expression of pOE33, pOE23, iOE23 and pLHCP in E.coli

Escherichia coli cells harboring the appropriate expression plasmids were grown in M9 media plus 0.5% tryptone to an OD at 600 nm of ~1.0. Isopropyl thio-β-D-galactoside (IPTG) was added to 1 mM and the cells were harvested by centrifugation after an additional 4 h at 37°C. For over-expression of ^{3}H-labeled protein, cells were grown in M9 medium to an OD at 600 nm of 0.2; cells were then pelleted, washed with M9 medium and resuspended in fresh M9 medium to 1/10 the original volume. IPTG was added to 1 mM, followed after 40 min by [^{3}H]leucine (~150 Ci/mmol) at 0.1 mCi/ml of culture. Cells were harvested by centrifugation after an additional 4 h at 37°C.

Inclusion bodies were isolated essentially by the method of Lin and Cheng (1991) with the following modifications. Cell pellets from 10 ml of a culture of unlabeled cells or 5 ml of a culture of ^{3}H-labeled cells were resuspended in 5 ml of 20 mM Tris–HCl (pH 7.5), 20% sucrose, 1 mM EDTA. After 10 min on ice, cells were pelleted at 3200 *g* for 10 min and resuspended in 2.5 ml of ice-cold water containing 1 mM phenyl methyl sulfonyl fluoride, 20 μg/ml aprotinin, 1 μg/ml leupeptin and, in the case of pOE33, lysozyme at 2 mg/ml. After 10 min on ice, one volume of 2 × phosphate-buffered saline was added and the suspension was frozen at −20°C overnight. The suspension was thawed and then passed through a Yeda press three times at 2500 p.s.i. Inclusion bodies were pelleted at 15 000 *g* for 20 min, washed three times with 1 ml phosphate-buffered saline, containing 5 mM EDTA, 25% sucrose and 1% Triton X-100, and three times with 1 ml cold water. Inclusion bodies were suspended in water and stored at −80°C. The specific radioactivity of the precursor proteins was determined from protein assay and scintillation counting of inclusion bodies solubilized with SDS. This method was verified for one preparation by determining the specific radioactivity of the protein band on a gel by a combination of comparison to Coomassie-stained bovine serum albumin standards and by extracting the proteins from the gel bands and scintillation counting. Specific radioactivities ranging from 400 000 to 900 000 d.p.m./μg protein were obtained with this method. *Escherichia coli*-produced proteins were prepared for import and thylakoid translocation assays by dissolving inclusion bodies in 8 M urea, 8 mM dithiothreitol (DTT) for ~4 h at room temperature. Unless otherwise specified, precursors were then pre-diluted with cold import buffer [50 mM HEPES/KOH (pH 8), 0.33 M sorbitol], 6 mM DTT to urea concentrations < 1 M urea and were added to reaction mixtures within 5 min of dilution. The final concentration of urea in import and thylakoid translocation assays was always < 0.4 M. Our analyses have shown that urea concentrations < 0.5 M do not significantly affect these assays.

Preparation of chloroplasts, lysates, thylakoids and stroma

Intact chloroplasts were isolated from 9- to 10-day-old pea (*Pisum sativum* L. cv. Laxton's Progress 9) seedlings (Cline, 1986). Chloroplasts were resuspended in import buffer and kept on ice until use. Chloroplast lysates were prepared from intact chloroplasts by osmotic lysis in 10 mM HEPES/KOH (pH 8.0) containing 10 mM $MgCl_2$ at 0°C with subsequent adjustment to import buffer containing 10 mM $MgCl_2$ (Cline, 1988). Stroma and thylakoids were separated by centrifuging lysates at 3200 *g* for 8 min at 4°C. Stroma was subjected to further centrifugation at 40 000 *g* for 30 min before use. Thylakoids were washed at least twice with import buffer containing 10 mM $MgCl_2$ before use.

Chloroplast protein import and thylakoid protein transport assays

Import into intact chloroplasts was carried out in microcentrifuge tubes in a 25°C water bath illuminated with 70 μE m^{-2} s^{-1} white light for 10 min (Cline *et al.*, 1989). Assays were generally conducted in a volume of 300 μl containing 100 μg chlorophyll of intact chloroplasts and 5 mM Mg-ATP. Assays were terminated by thermolysin treatment and repurification on Percoll cushions. Unless otherwise stated, assays for integration of LHCP into isolated thylakoids were conducted as described previously (Cline *et al.*, 1989). Briefly, radiolabeled pLHCP was incubated with chloroplast lysate or reconstituted lysate (thylakoids plus stromal extract) equivalent to 100 μg chlorophyll in a total volume of 300 μl of import buffer containing 10 mM $MgCl_2$. Reactions were conducted for the designated times at 25°C

in an illuminated water bath and terminated by transfer to 0°C, recovery of thylakoids by centrifugation, and subsequent thermolysin treatment to remove non-inserted LHCP. Integration was assessed by the appearance of LHCP-DP, a protease-protected LHCP peptide that is characteristic of properly inserted LHCP (Cline, 1986).

Unless otherwise stated, assays for transport of OE33, OE23, OE17 and PC into isolated thylakoids were conducted essentially as integration reactions with thylakoids alone or supplemented with stromal extracts, with or without Mg-ATP as described previously (Cline *et al.*, 1992) with the modifications specified in the text and figure legends. Following the assay, thermolysin post-treated membranes were washed once with 1 ml of import buffer containing 5 mM EDTA to remove the last traces of thermolysin prior to dissociation of the sample with SDS–PAGE buffer.

Analysis of samples

Samples recovered from the above assays were subjected to SDS–PAGE and fluorography (Cline, 1986). Quantification of the amount of import, integration or transport was accomplished by scintillation counting of radiolabeled proteins extracted from excised gel bands (Cline, 1986). The number of molecules of pLHCP, LHCP, LHCP-DP, pOE23, iOE23 and OE23 was determined from d.p.m. of the extracted bands, and the specific radioactivity of proteins used in the assays (Cline, 1986) and are reported as molecules/chloroplasts or chloroplast equivalent. Chloroplasts were quantified by chlorophyll determinations. There are 10^6 pea chloroplasts/μg chlorophyll.

Miscellaneous

Protein assays were performed by the BCA method (Pierce) using bovine serum albumin as a standard. Chlorophyll was determined by Arnon (1949). For analysis of the kinetic constants for protein import and transport, the average substrate concentration [*S*] and average reaction velocity, *V*, were calculated according to Lee and Wilson (1971). This procedure enables the Michaelis–Menten equation to be used with $<5\%$ error when up to 50% of the substrate has been depleted. Less than 5% pOE23 depletion occurred in transport assays and $<45\%$ pOE23 depletion in import assays. Less than 20% pLHCP depletion occurred in integration assays. K_m and V_{max} values were then determined from Eadie–Hofstee plots of the data. The K_m values are the average of two independent measurements. V_{max} values varied with the preparation and are reported for the experiments shown in the figures. For gel filtration chromatography of stromal intermediates, chloroplasts recovered from an import assay were lysed by resuspension in 100 μl of 10 mM HEPES/KOH (pH 8), 10 mM $MgCl_2$, followed by 100 μl of 2 × import buffer, 10 mM $MgCl_2$. The lysed chloroplasts were centrifuged at 40 000 *g* for 15 min. The supernatant was applied to a Superose 6 HR 10/30 and eluted with 20 mM HEPES/KOH (pH 8), 65 mM KCl, 1% (v/v) ethylene glycol.

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