The EMBO Journal vol.8 no.8 pp.2251 – 2255, 1989

ATP-dependent import of a lumenal protein by isolated thylakoid vesicles

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Communicated by R.J.Ellis

The 33 kd protein of the photosynthetic oxygen-evolving complex is synthesized in the cytoplasm as a larger precursor and transported into the thylakoid lumen via a stromal intermediate form. In this report we describe a reconstituted system in which the later stages of this import pathway can be studied in isolation. We demonstrate import of the 33 kd protein, probably as the intermediate form, into isolated pea thylakoids by a mechanism which is stimulated by the addition of ATP. The imported protein is processed to the mature size and is resistant to digestion by proteases. The thylakoidal protein transport system is specific in that non-chloroplast proteins and precursors of stromal proteins are not imported.

Key words: chloroplast protein transport/precursor proteins/processing/thylakoid lumen proteins

Introduction

A striking feature of chloroplast biogenesis is the complexity of the protein traffic into, and within, the organelle. The chloroplast is highly structured, consisting of a double membrane envelope which encloses the soluble stromal phase, and containing an internal thylakoid network which in turn encloses the thylakoid lumen. Most chloroplast proteins are encoded by nuclear genes and are transported into the appropriate compartment after synthesis in the cytoplasm as larger precursors (Schmidt and Mishkind, 1986; Ellis and Robinson, 1987). Transport into the chloroplast is post-translational and ATP-dependent (Grossman *et al.*, 1980; Flügge and Hinz, 1986).

Of particular interest is the biogenesis of cytoplasmically-synthesized thylakoid lumen proteins, since these proteins must cross all three chloroplast membranes to reach their sites of function. *In vitro* reconstitution studies on one such protein, plastocyanin, showed that the import of this protein can be divided into two phases. Pre-plastocyanin is initially imported into the stroma and processed to an intermediate form by a stromal peptidase which is highly specific for imported precursors. The import intermediate is then transferred across the thylakoid membrane and processed to the mature size by a second, thylakoidal peptidase (Robinson and Ellis, 1984; Hageman *et al.*, 1986; Smeekens *et al.*, 1986). Analysis of the partially purified thylakoidal peptidase has shown that this enzyme also displays a high degree of reaction specificity (Kirwin *et al.*, 1987, 1988).

Other thylakoid lumen proteins are believed to follow a similar import pathway. For example, the 33, 23 and 16 kd proteins of the photosynthetic oxygen-evolving complex (OEC) are initially synthesized with pre-sequences which resemble that of plastocyanin in terms of overall structure: a hydrophilic, positively-charged 'envelope transfer' domain, followed by a more hydrophobic 'thylakoid transfer' domain which is thought to be involved in transfer into the thylakoids (Jansen *et al.*, 1987; Tyagi *et al.*, 1987). In addition, we have recently shown that precursors of the 33 and 23 kd OEC proteins are processed to the intermediate and mature sizes by the partially purified stromal and thylakoidal processing peptidases, respectively (R.G.Herrmann and C.Robinson, unpublished data).

Although some of the basic features of chloroplast protein transport are now well-established, many of the mechanisms involved are largely unknown. In particular, little information is available to explain how proteins traverse the thylakoid membrane, since this event cannot be studied in isolation using the standard intact chloroplast import assay. In this report, we demonstrate that isolated thykaloid vesicles are capable of importing the 33 kd OEC protein by a mechanism which is stimulated by ATP.

Results

In vitro synthesis and maturation of wheat pre-33 kd

The lumenal protein used in this study is the 33 kd OEC protein of the photosynthetic oxygen-evolving complex. This protein is loosely attached to the lumenal face of the thylakoid membrane (Andersson *et al.*, 1984), where it appears to stabilize the manganese involved in water oxidation (Miyao and Murata, 1985). The precursor of the wheat protein (pre-33 kd) was synthesized by *in vitro* transcription–translation of a full-length cDNA clone to yield a polypeptide of M_r 41 000. This precursor is imported into the thylakoids of isolated pea chloroplasts and processed to the mature size (Figure 1A). The individual processing steps can be analysed in more detail using partially purified preparations of the peptidases involved; pre-33 kd is converted to an intermediate form by the stromal processing peptidase, supplied either as a crude stromal extract or after 350-fold purification, and is processed to the mature size by the thylakoidal peptidase (Figure 1B).

Protein import by isolated thylakoids

The reconstitution of the thylakoidal protein transport step was achieved by incubation of pre-33 kd with stromal extract, thylakoids and ATP. Under these conditions, pre-33 kd is converted to the intermediate form (by the stromal processing activity) and also to the mature size (Figure 2). Protease-protection assays show that the mature-size form is located inside the thylakoid vesicles: addition of thermolysin leads to the degradation of the precursor and intermediate forms

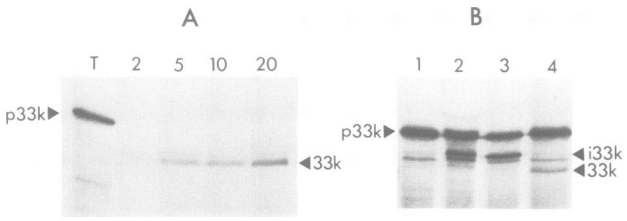

Fig. 1. *In vitro* synthesis and maturation of wheat pre-33 kd. **A:** pre-33 kd (**lane T**) was synthesized by transcription−translation of cDNA, and incubated with intact pea chloroplasts as described in Materials and methods. After incubation times given above the lanes (in min) the chloroplasts were treated with thermolysin, after which the thylakoids were isolated and analysed by SDS−polyacrylamide gel electrophoresis and fluorography. **B**: pre-33 kd (**lane 1**) was incubated with crude stromal extract (**lane 2**) partially purified stromal processing peptidase (**lane 3**) or thylakoidal processing peptidase (**lane 4**) as described in Materials and methods. Symbols: p33K; i33K; precursor and intermediate form of 33 kd protein. 33 kd: migration of authentic purified wheat 33 kd protein.

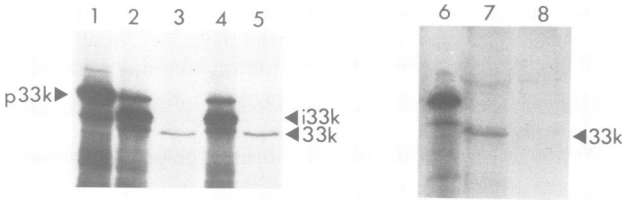

Fig. 2. Import of 33 kd protein by isolated pea thylakoids. Pre-33 kd (**lanes 1** and **6**) was incubated with thylakoids, stroma, and ATP in the presence (**lanes 2** and **3**) and absence (**lanes 4** and **5**) of 300 mM sorbitol. After incubation as described in Materials and methods, samples were analysed without further treatment (lanes 2 and 4) or after thermolysin treatment of the thylakoids (lanes 3 and 5). Sonication control: after import incubations, thylakoids were incubated with thermolysin (**lane 7**) or were incubated with thermolysin and were sonicated during the incubation period (**lane 8**). At the end of the incubation period, EDTA was added to 50 mM, 1 vol sample buffer was added, and the samples were boiled for 2 min. Symbols as in Figure 1.

but most of the mature 33 kd is resistant unless the thylakoids are sonicated during the thermolysin treatment to allow access of the protease into the lumen. Some mature-size, protease-accessible 33 kd is often generated in this assay system; this may be due to non-specific proteolysis by stromal or thylakoidal peptidases, or this may indicate a sub-population of 33 kd molecules which have been partially transported across the thylakoid membrane and processed to the mature size. It is unlikely that mature 33 kd leaks out from the thylakoids, since the vesicles are usually very tightly sealed under these conditions. It has been reported that thylakoids undergo spontaneous vesiculation during prolonged incubation in hypotonic media (Andersson and Anderson, 1985). However, Figure 2 shows that the rates of both processing of pre-33 kd to the intermediate form, and import into the thylakoids, are similar in the presence and absence of 300 mM sorbitol, indicating that isotonic conditions are not required in this system.

It should be emphasized that protein import by intact chloroplasts in the incubation mixture can be ruled out; complete lysis of the organelles is routinely verified by a Percoll pad procedure (Cline, 1986) and by phase-contrast microscopy. Furthermore, the protein import capacity of the thylakoids is unimpaired by several rounds of freeze−thawing, a procedure which quantitatively lyses chloroplasts.

The effects of omitting stromal extracts or ATP from the uptake incubation mixtures are shown in Figure 3. In this

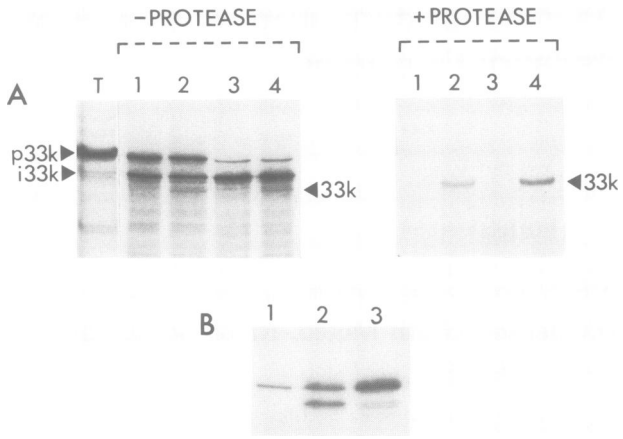

Fig. 3. Effects of added stromal extract and ATP on the import of 33 kd by isolated thylakoids. **A**: Thylakoids were isolated as described in Materials and methods except that they were also washed twice in 50 mM Tricine−KOH, pH 8.0, 2 M NaBr before resuspension either in 100 mM Tricine−KOH, pH 8.0, or stromal extract. Pre-33 kd (lane T) was incubated with thylakoids in Tricine−KOH (**lanes 1** and **2**) or stromal extracts (**lanes 3** and **4**). Lanes 1 and 3; ATP was omitted from the incubation mixtures. Samples were analysed before or after thermolysin treatment as indicated above the lanes; incubation conditions and protease treatments were as Figure 2 except where stromal extract or ATP was omitted. **B**: pre-33 K (**lane 1**) was dialysed against 100 mM Tricine−KOH, pH 8.0 for 3 h at 4°C (**lane 2**) or against the same buffer containing 10 mM EDTA (**lane 3**).

experiment, import assays were carried out using thylakoids which had been washed twice in 2 M NaBr, a drastic procedure which removes not only stromal proteins but also some extrinsic thylakoid proteins (Westhoff *et al.*, 1985). A surprising finding was that, even in the apparent absence of stroma, pre-33 kd is efficiently processed to the intermediate form and, in the presence of ATP, imported into the thylakoids (Figure 3A, lanes 2). The rates of cleavage and import are greater in the presence of added stromal extract, but only by a factor of about 2 (lanes 4). Control tests have shown that, in the absence of added chloroplast stroma, cleavage of pre-33 kd to the intermediate size is carried out by stromal processing activity present in the translation mix. Processing of pre-33 kd can be achieved by relatively brief dialysis of the translation mix against higher pH buffer (Figure 3B) and this processing is inhibited in the presence of EDTA, a compound which is known to inhibit the stromal processing peptidase (Robinson and Ellis, 1984). The processing activity, which probably originates from proplastids in the wheatgerm embryos, is virtually inactive during the translation incubation but appears to be activated by the higher pH conditions of the import mixture. The levels of processing activity in the wheatgerm extract are, in fact, relatively low: the efficient processing observed in lanes 1 and 2 reflects the fact that wheat pre-33 kd is an excellent substrate for the stromal enzyme. We have found that this precursor is processed at a rate 5- to 10-fold higher than any other precursor tested, including spinach pre-33 kd (unpublished data).

A more detailed examination of the effects of increasing concentrations of ATP on the rate of import of 33 kd protein is shown in Figure 4. The optimal concentration of ATP under these assay conditions is 10 mM; higher concentrations inhibit both processing by the stromal peptidase and import into the thylakoids. Quantitation of the data shown in Figure 4, by scintillation counting of excised bands, indicates

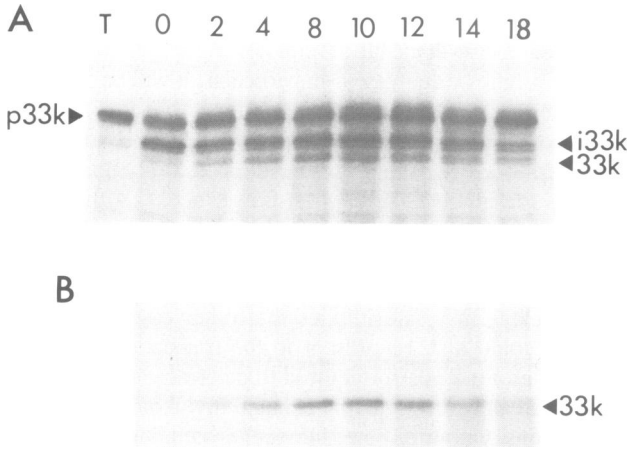

Fig. 4. ATP-dependence of import into thylakoids. Pre-33 kd was incubated with thylakoids and stroma, as described in Materials and methods, and ATP at concentrations (in mM) given above the lanes. Samples were analysed before (**A**) or after (**B**) thermolysin treatment. Symbols as in Figure 1.

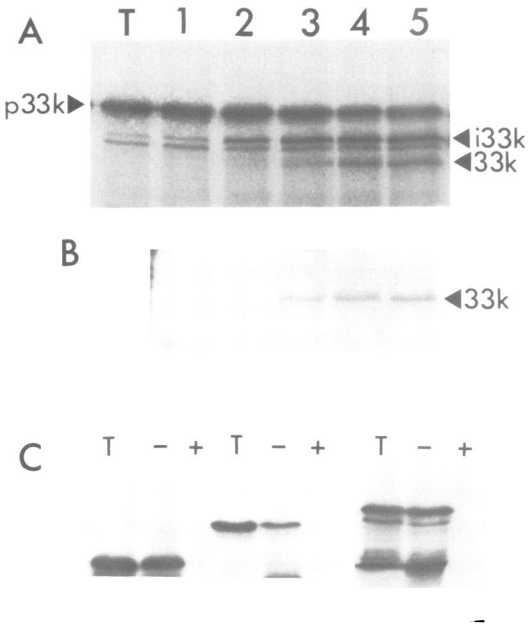

Fig. 5. Effects of inhibition of the stromal peptidase on the import of 33 kd protein by isolated thylakoids. Pre-33 kd was incubated with thylakoids, stroma and ATP as described in Materials and methods. Incubation mixtures contained a 20mer peptide (see text) at 5 mM (**lane 1**), 1 mM (**lane 2**), 0.2 mM (**lane 3**) and 0.05 mM (**lane 4**) or no peptide (**lane 5**). **Lane T**, pre-33 kd translation product. Samples were analysed before (**A**) or after (**B**) protease treatment. Symbols as in Figure 1. **C**: tests for import of other proteins into thylakoids. Import incubations were carried out as in Figure 2 except that pre-33 Kd was replaced by (left to right) pre-lysozyme, pre-acyl carrier protein, and pre-Rubisco small subunit. After incubation, samples were analysed either before (−) or after (+) protease K treatment (200 μg/ml for 45 min at 4°C). **Lanes T** = translation products.

that 11% of total available pre-33 kd, or ~20% of processing intermediate, is imported in the presence of 10 mM ATP.

Specificity of the thylakoidal import system

The data shown in Figures 2 and 3 demonstrate that isolated thylakoids are capable of 33 kd import, but do not indicate whether it is the precursor or intermediate form of the 33 kd protein (or both) which is taken up. We attempted to resolve this point by inhibiting the stromal processing activity in the

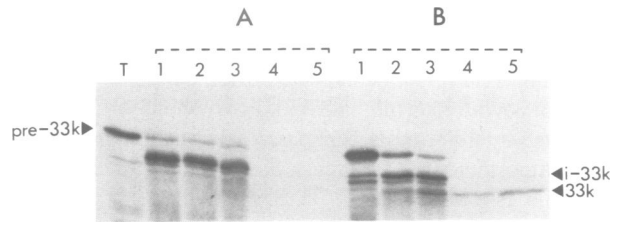

Fig. 6. Incubation of the 33 kd processing intermediate with isolated thylakoids. **Panel A**: incubation conditions were as in Figure 2 except that pre-33 kd, stroma, ATP and $MgCl_2$ (100 μl) were pre-incubated for 30 min at 27°C. A sample (20 μl) of this mixture was removed (**lane 1**) and the remainder was used to resuspend a thylakoid pellet (100 μg chlorophyll). Incubation was at 27°C for a further 15 and 30 min, at which times samples were analysed directly (**lanes 2** and **3**) or after thermolysin treatment (**lanes 4** and **5**). **Panel B**: pre-33 kd, stroma, ATP and $MgCl_2$ were mixed as in A except that a sample was immediately removed (**lane 1**) and the remainder used to resuspend thylakoids as rapidly as possible. After further incubation for 15 and 30 min, samples were analysed (as in A) directly (**lanes 2** and **3**) or after thermolysin treatment of the thylakoids (**lanes 4** and **5**).

import incubation mixture, in order to determine whether pre-33 kd can be imported under these conditions. This was achieved by including in the import assay a competitive inhibitor of the stromal peptidase; a 20mer peptide which corresponds to residues 23−42 of the pre-sequence of *Silene* pre-plastocyanin (Smeekens *et al.*, 1985). Processing by the stromal peptidase is believed to take place between residues 39 and 40, or in the near vicinity (Hageman *et al.*, 1986). Figure 5A shows that increasing concentrations of the peptide progressively inhibit both the processing of pre-33 kd to the intermediate form, and the appearance of mature-size 33 kd inside the thylakoids. This result suggests that isolated thylakoids are capable of importing the 33 kd intermediate, but not the full precursor. This suggestion is supported by other data; over the course of a number of import experiments, we have observed an overall correlation between the extent of processing of pre-33 kd to the intermediate, and the level of import into the lumen. It should, however, be emphasized that these data do not constitute conclusive evidence that thylakoids import only the intermediate form of the 33 kd protein. For example, the peptide used in Figure 5A to inhibit the stromal peptidase may also inhibit the translocation system.

Other tests on the specificity of the transport system have been carried out. As might be expected of a thylakoidal protein transport system, no import of foreign proteins (such as pre-lysozyme, a secreted protein) or precursors of stromal proteins such as acyl carrier protein or Rubisco small subunit is observed (Figure 5C).

A useful property of the 20mer peptide described in Figure 5 is that it does not appear to affect the envelope-based protein transport system, enabling us to distinguish experimentally between the activities of this system and the thylakoidal transport system. Thus, 5 mM peptide completely inhibits 33 kd import by isolated thylakoids (Figure 5) but has no apparent effect on the rate of import of precursor proteins (including pre-33 kd) by isolated chloroplasts, although their subsequent maturation is affected (J.Musgrove and C.Robinson, manuscript in preparation). These observations eliminate the possibility that intact chloroplasts could be responsible, even in part, for the protein transport events described in Figures 2−5.

On the basis of the results described above, and those of others (Hageman *et al.*, 1986; Smeekens *et al.*, 1986) it

appears likely that processing to the intermediate is required before a lumenal protein can be imported by thylakoids. Accordingly, it might be expected that incubation of isolated thylakoids with intermediate 33 kd, which has already been generated by the stromal peptidase, may result in particularly rapid import into the thylakoids. However, Figure 6A shows that this is not the case. Pre-33 kd is very efficiently processed to the intermediate form during incubation with stromal extract for 30 min at 27°C (lane 1) but virtually no import is observed when this mixture is subsequently incubated with thylakoids (lanes 2–5). In the control incubation (B) pre-33 kd was mixed with stromal extract, after which this mixture was incubated with thylakoids as rapidly as possible; under these conditions, efficient import of 33 kd protein by thylakoids is observed (lanes 2–5). It is notable that in the time required to take the 'zero time' sample for the control incubation (after mixing of pre-33 kd with stroma and then thylakoids; ~10–15 sec) significant processing to the intermediate takes place (lane 1 of Figure 5B). This observation is reproducible, and emphasizes the 'processability' of this precursor.

There are several possible explanations for the result shown in Figure 6. The first is that isolated thylakoids do not import 33 kd intermediate generated in this way because the natural substrate for the transport system is the full precursor. We cannot exclude this possibility, but, in our view, it is unlikely for reasons already given above. The second possibility is that thylakoids do import the intermediate, but that this form is labile once generated by a crude stromal extract. A third, related, possibility is that the intermediate is more stable, or import-competent, if generated in the presence of thylakoid membranes. Further studies are required to resolve these possibilities, and to define more precisely the factors required for protein transport across the thylakoid membrane.

Discussion

In this report we have shown that isolated thylakoid vesicles possess a protein transport activity capable of importing the 33 kd OEC protein into the lumenal space. The available evidence strongly suggests that thylakoids take up the processing intermediate form but not the complete precursor, which is consistent with the current model for the import pathway taken by cytoplasmically-synthesised lumenal proteins. The model proposes that the second, 'thylakoid transfer' domain of the pre-sequence is responsible for directing the import intermediate into the thylakoid lumen, after removal of the first domain by the stromal processing peptidase (Smeekens *et al.*, 1986).

The reconstituted system is reasonably efficient in that up to ~20% of the available intermediate can be imported by the thylakoids, and future work will aim to increase the efficiency of import. It is, for example, possible that the energy requirements of the import system have not been fully satisfied, since we have yet to analyse in detail the energetics of the protein transport mechanism. Our results suggest that the transport system may require energy in the form of ATP, as does protein transport across the envelope (Grossman *et al.*, 1980; Flügge and Hinz, 1986). However, it is presently unclear whether ATP is the sole requirement, the most effective source of energy, or even whether ATP is hydrolysed in this assay system.

The development of an *in vitro* assay for protein transport into the lumen will enable several key aspects of the transport mechanism to be addressed. In particular, it will be of interest to determine whether thylakoidal factors (for example, receptor/transport proteins) are involved in 33 kd import. Furthermore, we cannot exclude the possibility that stromal factors are involved in the transport process. Although we have shown that the addition of chloroplast stromal extracts is not a prerequisite for 33 kd transport across the thylakoid membrane, the detection of stromal processing activity in the wheatgerm lysate implies that other essential stromal factors could also be present in the translation mix.

Further work is also required to determine whether other proteins can be imported by isolated thylakoids under these conditions. Only one other lumenal protein has been tested, *Silene pratensis* pre-plastocyanin, and no import was observed (unpublished results). However, previous work on this protein suggests that it may be a poor substrate for this type of study. During import of pre-plastocyanin into isolated pea chloroplasts, a prominent stromal intermediate is observed, which is relatively slowly transferred into the thylakoids (Smeekens *et al.*, 1986). In contrast, no stromal intermediate has been detected during the import of wheat pre-33 kd into isolated pea chloroplasts (Figure 1) suggesting that the stromal intermediate is imported from the stroma into the thylakoids much more rapidly.

This may be an indication that thylakoids import different lumenal proteins at markedly different rates. Interestingly, a prominent stromal intermediate is observed during the import of spinach pre-33 kd into pea or spinach chloroplasts, suggesting that there may be variations between species in the efficiencies with which given proteins are imported by thylakoids (C.Robinson and R.G.Herrmann, unpublished data).

It is not clear precisely when processing of the 33 kd intermediate to the mature size occurs during the biogenesis of this protein, but studies on the thylakoidal processing peptidase have partially resolved the sequence of events during the later stages of 33 kd import. Firstly, the active site of the peptidase is located on the lumenal face of the thylakoid membrane, ruling out the possibility that processing occurs prior to the transport step. Secondly, the thylakoidal peptidase is located exclusively in non-appressed stromal lamellae of the thylakoid network (Kirwin *et al.*, 1988) whereas mature 33 kd protein is functional in the appressed granal lamellae, suggesting that maturation takes place before the protein reaches the granal lamellae. Finally, we have not been able to detect imported, unprocessed 33 kd intermediate in the thylakoid lumen using this assay system. Taken together, these observations indicate that the 33 kd intermediate is processed to the mature size either during, or very shortly after, transport across the thylakoid membrane.

Although the transport of proteins into the lumen of isolated thylakoids has not been previously reported, the integration of one protein into the thylakoid membrane has been analysed in some detail. It has been shown (Cline, 1986; Chitnis *et al.*, 1987) that the light-harvesting chlorophyll a/b protein can integrate into isolated thylakoids and assemble into Photosystem II. However, the mechanisms involved in the membrane integration of this protein, and the transport of 33 kd into the lumen, differ in two significant respects. Firstly, membrane integration of the chlorophyll a/b protein

is dependent on the presence of added chloroplast stromal extracts whereas 33 kd transport can take place in the absence of such an extract (although we cannot yet rule out the possible presence of essential translocation factors in the translation mix). Secondly, the information required for the membrane integration of the chlorophyll a/b protein is located in the mature protein sequence, and no processing intermediate is generated during import into the chloroplast (Lamppa, 1988; Viitanen *et al.*, 1988). In contrast, the thylakoid transfer domain of imported lumenal proteins is thought to mediate transport of the intermediate across the thylakoid membrane, and deletion of this domain from pre-plastocyanin abolishes transport into the lumen (Smeekens *et al.*, 1985). The integration of the light-harvesting chlorophyll a/b protein and the transport of lumenal proteins thus appear to be mediated by distinct mechanisms; further work is required to verify this hypothesis and to define in greater detail the processes involved in each case.

Materials and methods

Materials

Pea seedlings (*Pisum sativum* var. Feltham First) were grown under a 12 h photoperiod for 8 days as described (Blair and Ellis, 1973). Radioactive materials and Amplify were obtained from Amersham International (UK). Peptide synthesis was carried out by Dr T.Doel (Institute for Animal Health, Pirbright, UK).

cDNA cloning and expression

A λgtll cDNA expression library (kindly supplied by C.Raines and T.A.Dyer) was screened with a specific polyclonal antiserum raised against purified 33 kd protein from pea (*Pisum sativum*) chloroplasts. Purification of the 33 kd protein was as described (Westhoff *et al.*, 1985). One of the positives contained a 1300 bp insert which was subcloned into pGem4Z (Promega Biotech) giving rise to p33K-2. Nucleotide sequencing has shown that the predicted amino acid sequence is higly homologous to the corresponding spinach sequence determined by Tyagi *et al.* (1987) (data not shown). Pre-33 kd was synthesized by transcription of p33k-2 using SP6 RNA polymerase (Melton *et al.*, 1984) followed by translation of capped transcripts in a wheatgerm system (Anderson *et al.*, 1983) in the presence of [^{35}S]methionine.

Chloroplast protein import and processing studies

Processing of pre-33 kd involved incubation of 0.5 μl translation mix with 25 μl of partially purified stromal processing peptidase (Robinson and Ellis, 1984) or thylakoidal peptidase (Kirwin *et al.*, 1987). After incubation for 60 min at 27°C, reactions were stopped by the addition of 1 vol sample buffer [125 mM Tris−HCl, pH 6.8, 4% (w/v) SDS, 5% (w/v) sucrose, 5% (v/v) 2-mercaptoethanol] followed by boiling for 2 min. Samples were analysed by SDS−polyacrylamide gel electrophoresis (Laemmli, 1970) followed by fluorography using Amplify. Pre-33 kd was imported into intact pea chloroplasts as described for other precursors (Robinson and Ellis, 1985). After import, the chloroplasts were treated with thermolysin (200 μg/ul) for 20 min at 4°C before re-isolation and analysis by electrophoresis as above.

Thylakoid protein import studies. Intact pea chloroplasts were prepared by Percoll gradient centrifugation as described (Robinson and Ellis, 1985). The chloroplasts were lysed in 50 mM tricine−KOH, pH 8.0 for 20 min at 4°C, centrifuged at 5000 *g* for 5 min, and washed once in lysis buffer and twice in 10 mM Tricine−KOH, pH 8.0, 300 mM sorbitol, 5 mM $MgCl_2$ before resuspension in the stromal supernatant. Import incubation mixtures (40 μl) contained thykaloids (25 μg chlorophyll), stromal extract (30 μg protein), 5 μl wheatgerm translation mix containing pre-33 kd, 10 mM ATP and 10 mM $MgCl_2$. Incubation was for 60 min unless otherwise specified, after which half of the sample was removed and boiled in sample buffer as above. The remaining 20 μl were incubated with thermolysin (400 μg/ml) for 30 min on ice, then diluted with 1 ml of 100 mM Tricine−KOH, 50 mM EDTA pH 8.0, and the thylakoids were pelleted by centrifugation for 10 min in a microfuge. The thylakoids were washed with 1 ml of the same buffer, then resuspended in 20 μl of the buffer, mixed with sample buffer and boiled for 2 min. Samples were analysed by electrophoresis and fluorography as above.

Other methods. Published methods were used for the determination of chlorophyll (Arnon, 1949) and protein (Bradford, 1976).

Acknowledgements

This work was supported by SERC grants GR/D90390 and GR/D81756.

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Received on February 20, 1989; revised on May 5, 1989