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The presequence of a chimeric construct dictates which of two mechanisms are utilized for translocation across the thylakoid membrane: evidence for the existence of two distinct translocation systems

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The translocation of plastocyanin across the thylakoid membrane in *Pisum sativum* has been studied in reconstitution assays and using chimeric constructs. The reconstitution assays demonstrate that plastocyanin translocation is absolutely dependent on the presence of a stromal factor(s) and nucleotide triphosphates (NTPs), whereas neither element is required for the translocation of the 23 or 16 kDa proteins of the oxygen-evolving complex. Previous studies had revealed that the transthylakoidal delta pH is essential for translocation of the 23 and 16 kDa proteins but unnecessary for plastocyanin translocation. The basis for these mechanistic differences has been tested by analysing the translocation of a chimeric construct consisting of the presequence of the 23 kDa protein linked to the mature plastocyanin sequence. This construct is efficiently imported into thylakoids in the absence of stromal extracts or NTPs and translocation across the thylakoid membrane within intact chloroplasts is totally inhibited by the uncoupler nigericin: the translocation requirements are thus identical to those of the pre-23 kDa protein and diametrically opposite to those of pre-plastocyanin. Transport across the thylakoid membrane of a second fusion protein, consisting of the presequence of the 16 kDa protein linked to mature plastocyanin, is also dependent on a delta pH. The data suggest that two distinct systems are involved in the translocation of proteins across the thylakoid membrane, with each system recognizing specific signals within the presequences of a subset of lumenal protein precursors.

Key words: chloroplast/plastocyanin/protein transport/thylakoid biogenesis

Introduction

Considerable attention has centred on the biogenesis of cytosolically synthesized thylakoid proteins, in an attempt to determine how the correct intraorganellar 'sorting' of these proteins is achieved after import into the chloroplast. The biogenesis of hydrophilic thylakoid lumen proteins is especially intriguing, since these must cross both the soluble stromal phase and the thylakoid membrane after import into the chloroplast. A variety of *in vitro* studies on four prominent lumenal proteins: plastocyanin and the 33, 23 and 16 kDa proteins (33K, 23K and 16K) of the oxygen-evolving complex, have indicated that this complex import pathway can be resolved into two principal phases. The first phase involves the import of a cytosolically synthesized precursor protein across the envelope membranes and subsequent cleavage to an intermediate size form by a stromal processing peptidase. The intermediate form is then transported across the thylakoid membrane and cleaved to the mature size by a thylakoidal processing peptidase (Hageman *et al.*, 1986; Smeekens *et al.*, 1986; James *et al.*, 1989; Clausmeyer *et al.*, 1993). *In vivo* pulse labelling studies on the biogenesis of lumenal proteins in *Chlamydomonas reinhardtii* have provided additional evidence for a two-step import mechanism (Howe and Merchant, 1993).

In keeping with this import pathway, there is compelling evidence that the presequences of lumenal proteins contain two distinct targeting signals in tandem, specifying 'envelope transit' and 'thylakoid transfer'. The transit signals, which are hydrophilic, basic and enriched in hydroxylated residues, are structurally and functionally equivalent to the presequences of imported stromal proteins (Ko and Cashmore, 1989; von Heijne *et al.*, 1989; Hageman *et al.*, 1990). In contrast, thylakoid transfer signals are strikingly similar in structural terms to signal sequences which direct the translocation of proteins across the endoplasmic reticulum and the bacterial plasma membrane (von Heijne *et al.*, 1989). Functional similarities between the two types of peptide have also been demonstrated by the ability of *Escherichia coli* to export pre-33K from spinach and wheat (Seidler and Michel, 1990; Meadows and Robinson, 1991), and pre-plastocyanin from spinach (W.Haehnel, T.Jansen, K.Gause, R.B.Klösgen, B.Stahl, D.Michl, B.Huvermann, M.Karas and R.G.Herrmann, manuscript submitted). *E.coli* signal peptidase and pea thylakoidal processing peptidase have also been shown to possess virtually identical reaction specificities (Halpin *et al.*, 1989). In view of these similarities, it is widely believed that the thylakoidal protein transport apparatus evolved from a translocation system in a cyanobacterial-type progenitor of the chloroplast.

The characteristics of the thylakoidal protein transport apparatus in higher plant chloroplasts have been examined using *in vitro* import assays in which precursor proteins are incubated with isolated thylakoids. Most of these studies have used the precursors of 33K, 23K and 16K as substrates, since these proteins were imported with high efficiency. The results indicated that the transthylakoidal delta pH is essential for the translocation of 23K and 16K, and has a stimulatory effect on the translocation of 33K (Mould and Robinson, 1991; Mould *et al.*, 1991; Cline *et al.*, 1992; Klösgen *et al.*, 1992). In addition, Cline *et al.* (1992) demonstrated that the import of 23K and 16K into thylakoids did not require the presence of nucleotide triphosphates (NTPs). In contrast, it has recently been found that the import of 33K into

thylakoids is strictly dependent on the presence of NTPs and requires in addition the presence of at least one stromal protein factor (Hulford *et al.*, 1994). Lumenal proteins are thus translocated across the thylakoid membrane by (at least) two radically distinct mechanisms.

It has been more difficult to establish details of the mechanism by which plastocyanin is transported across the thylakoid membrane, because the pea and *Arabidopsis* precursor proteins were found to be imported very inefficiently by isolated thylakoids (Bauerle and Keegstra, 1991). However, Theg *et al.* (1989) observed that plastocyanin could be efficiently transported into the lumen of intact chloroplasts in the complete absence of a thylakoidal proton motive force, which has been taken as evidence that this protein is translocated by a mechanism radically different to either of those utilized by the oxygen-evolving complex proteins. In this report we show that spinach pre-plastocyanin can be efficiently imported into isolated pea thylakoids by a mechanism which requires the presence of both a stromal protein factor and NTPs. We furthermore show that the translocation of a codon-correct fusion protein, consisting of the 23K presequence followed by the plastocyanin mature sequence, requires a delta pH but that stromal factors and NTPs are completely unnecessary; the translocation mechanism is thus apparently identical to that of 23K and 16K. The data suggest that two distinct translocation systems are involved in the translocation of proteins across the thylakoid membrane, each recognizing specific features in the presequences of a subset of lumenal proteins.

Results

Import of spinach pre-plastocyanin into isolated thylakoids requires a stromal factor

In an effort to analyse the mechanism by which plastocyanin is transported across the thylakoid membrane, the precursor of the spinach protein was synthesized by *in vitro* transcription–translation and incubated with isolated pea thylakoids in an import assay based on that of Mould *et al.* (1991). Figure 1A shows that after incubation of pre-plastocyanin with thylakoids in the presence of stromal extract, and subsequent protease treatment of the thylakoids, mature size plastocyanin is present in significant amounts (lane 2) whereas virtually no import is apparent in the absence of stromal extract (lane 4). The data are complicated by the presence in the translation products of a polypeptide of identical mobility to mature size plastocyanin. However, it is clear that incubation with thylakoids in the presence of stromal extract leads to a significant decrease in the amount of precursor protein, with a corresponding increase in the amount of the mature size protein in lane 1. Calculation of the import efficiency by densitometric scanning (taking into account the loss of two out of four labelled methionine residues during the conversion of pre-plastocyanin to the mature size), reveals that the amount of protease-protected plastocyanin in lane 2 is equivalent to 27% of available precursor. This is considerably higher than was observed in tests using the pea and *Arabidopsis* precursors, in which import efficiencies of only 1–6% were observed (Bauerle and Keegstra, 1991).

In order to confirm that import into the lumen had taken place, attempts were made to demonstrate that the imported plastocyanin is sensitive to proteolysis when the thylakoids

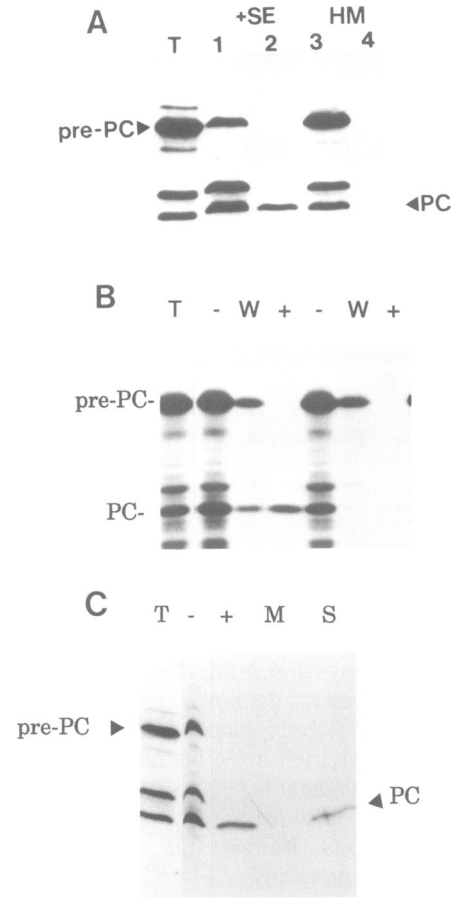

Fig. 1. Import of pre-plastocyanin into isolated pea thylakoids requires the presence of stromal extract. (**A**) Pre-plastocyanin was synthesized by *in vitro* transcription–translation and incubated with washed pea thylakoids which had been suspended in stromal extract (SE; lanes 1 and 2) or HM buffer (lanes 3 and 4). After incubation, samples were analysed directly (lanes 1 and 3) or after protease treatment of the thylakoids (lanes 2 and 4). (**B**) Import assays were carried out in the presence of stromal extract or HM buffer (SE, HM), after which samples were analysed directly, after washing of the thylakoids twice in 2 M NaBr or after protease treatment of the thylakoids (lanes denoted −, W, +, respectively). (**C**) Import was carried out in the presence of stromal extract, after which samples were analysed directly or after protease treatment of the thylakoids (lanes −, +). A further sample was treated with protease, after which the thylakoids were washed once in HM buffer and the sample was sonicated for 5 s. The sample was then centrifuged for 15 min in a microfuge and supernatant (lane S) and membrane samples (lane M) were analysed. Pre-PC, precursor of plastocyanin; PC, mobility of mature size plastocyanin; lane T, translation product.

are disrupted, for example by detergents. However, we have found that mature plastocyanin is extremely resistant to proteolysis, such that the concentrations of thermolysin or trypsin required for complete degradation are able to disrupt the thylakoid membrane even in the absence of detergent (not shown). We therefore used another technique to rule out the possibility that the mature size, protease-protected plastocyanin is bound to the stromal face of the thylakoid membrane, in which extrinsic proteins were removed by washing the thylakoids in 2 M NaBr after the import incubation. The results (Figure 1B) show that when thylakoids are washed after incubation with pre-plastocyanin in the absence of stromal extract, no mature size plastocyanin remains with the thylakoids, although a small amount of precursor remains bound to the stromal surface. When the

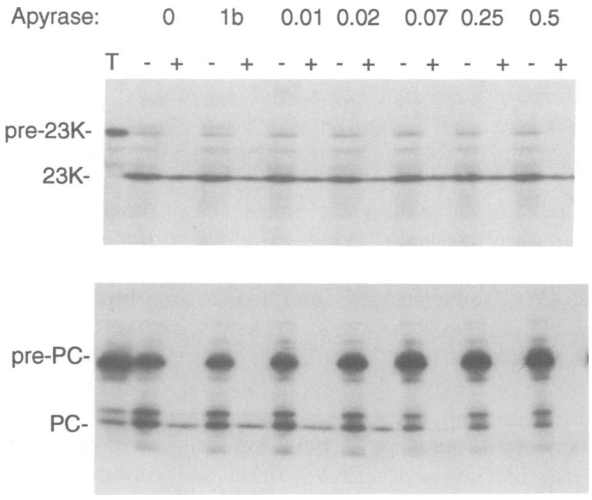

Fig. 2. NTPs are essential for the import of plastocyanin, but not 23K, into isolated thylakoids. Thylakoid import assays using pre-23K (upper panel) or pre-plastocyanin (lower panel) were carried out in the presence of stromal extract as detailed in Materials and methods, except that the mixtures of translation product, stromal extract and thylakoids were preincubated with apyrase (units of activity per incubation mixture indicated above the lanes) for 10 min on ice before incubation at 25°C. In the control denoted 1b, boiled apyrase was added. After the import incubations, samples were analysed directly or after protease treatment (−, +).

import incubation is carried out in the presence of stromal extract, a similar quantity of mature size protein is resistant both to NaBr washing and to protease treatment of the thylakoids. These data strongly suggest that the mature size, protease-protected plastocyanin is located in the thylakoid lumen. The remote possibility exists that this species of polypeptide is both protease resistant and tightly bound to the stromal face of the thylakoid membrane. However, Figure 1C shows that the mature size, protease-protected plastocyanin is quantitatively released from the thylakoids following a brief sonication, indicating that translocation into the lumen has indeed taken place and that the protease treatment effectively removes non-imported proteins.

Whereas the precursor of *Silene pratensis* plastocyanin is processed to the intermediate form by stromal processing peptidase (SPP) activity in stromal extracts (Hageman *et al.*, 1986), no cleavage of the spinach precursor has been detected after incubation with stromal extracts, either during the thylakoid import assays (Figure 1) or in tests using partially purified SPP (data not shown). This does not necessarily imply that cleavage does not take place *in vivo*, because a significant proportion of known substrates are not processed by SPP in organelle-free assays, for unknown reasons (Musgrove *et al.*, 1989). Furthermore, Clausmeyer *et al.* (1993) have shown that the spinach plastocyanin presequence can be cleaved at an intermediate site during import into isolated chloroplasts. We can, however, conclude that the full precursor protein is being translocated across the thylakoid membrane in this *in vitro* assay and that the stromal factor required for plastocyanin translocation is not SPP. Other studies have shown that the full precursors of 23K and 16K can also be transported across the thylakoid membrane with high efficiency (Mould *et al.*, 1991; Cline *et al.*, 1992; Klösgen *et al.*, 1992), suggesting that in most cases, cleavage by the stromal peptidase is not an important step in the import pathway.

NTPs are essential for the import of pre-plastocyanin into isolated thylakoids

Since recent work has shown that the import of 33K into thylakoids requires the presence of both a stromal factor and NTPs, we were interested in testing whether the same applied to plastocyanin import. Figure 2 shows the effects on import of pretreating the translation mixture and stromal extract with apyrase, which hydrolyses NTPs. For comparison, apyrase was incubated with assay mixtures containing pre-23K, since Cline *et al.* (1992) have shown that apyrase treatment does not affect the import of this precursor into thylakoids. The results show that as expected, apyrase treatment has no effect on the import of pre-23K; efficient import is observed in all incubations. In striking contrast, concentrations of apyrase >0.25 units/incubation mixture completely inhibit the import of pre-plastocyanin into thylakoids, indicating that NTPs are absolutely essential for the translocation process. It is highly likely that import is dependent on ATP, since this is the dominant NTP in the translation mixture and is present at ~ 0.15 mM in the import assay. However, we cannot as yet rule out the possibility that other NTPs are involved in the translocation process.

A 23K−PC fusion protein is translocated across the thylakoid membrane by a mechanism similar to that of pre-23K and not pre-plastocyanin

On the basis of the data presented above and from other studies, it is clear that plastocyanin is transported across the thylakoid membrane by a mechanism which differs from that of 23K and 16K in three important respects: (i) a stromal factor is required for import into isolated thylakoids, (ii) NTPs are required for import into thylakoids and (iii) a delta pH is unnecessary for translocation across the thylakoid membrane in intact chloroplasts. These findings prompt the critical question: do these differences reflect the existence of two distinct translocation systems, or are they a consequence of the differing properties of the mature proteins being translocated? If these proteins are transported by two distinct translocases, such systems would be expected to be able to discriminate between the thylakoid transfer signals of plastocyanin or 23K, since the targeting signals are believed to reside in these peptide extensions. On the other hand, it is possible that a stromal factor and ATP are required to maintain the plastocyanin (and 33K) mature proteins in an unfolded, translocation-competent conformation; 23K and 16K may instead adopt more flexible conformations which enable these proteins to bypass such factors. The differing responses of these proteins to uncouplers could similarly be explained by either of two scenarios: (i) plastocyanin is transported by a separate transport system which utilizes a stromal factor and NTPs, but not a delta pH or (ii) the three proteins are all transported by the same system, but the characteristics (for example, charge distribution) of the plastocyanin mature protein are such that a delta pH is not required for translocation across the thylakoid membrane. We have addressed these questions by examining the translocation of a chimeric protein, 23K−PC (PC stands for plastocyanin), consisting of the spinach 23K presequence linked to mature size plastocyanin, which has been shown by Clausmeyer *et al.* (1993) to be efficiently imported into intact chloroplasts and transported into the thylakoid lumen. Although most of the previous work on the transport of pre-23K across the thylakoid membrane has been carried out using the wheat or pea precursor proteins, control tests

(not shown) have confirmed that in both chloroplast import and thylakoid import assays, identical results are obtained with spinach pre-23K.

Figure 3 details the results of thylakoid import assays using 23K−PC as a substrate. In both the presence and absence of stromal extract, incubation with isolated thylakoids results in the generation of mature size plastocyanin which is resistant to protease treatment. Import is slightly more efficient in the absence of stromal extracts, indicating that stromal factors are not required for the import of this construct into thylakoids. We have similarly observed that import of pre-23K is often more efficient in the absence of stromal extracts (Mould *et al.*, 1991).

The effects of apyrase on the import of 23K−PC into thylakoids are shown in Figure 4. Import assays were conducted in the presence of stromal extract to ensure that the results were comparable with the control assays using plastocyanin as a substrate. The results show that prior treatment of the import mixtures with a variety of concentrations of apyrase has no significant effect on the import of 23K−PC into thylakoids, whereas in the control tests (not shown), import of pre-plastocyanin was completely inhibited by the higher concentations of apyrase as demonstrated in Figure 2.

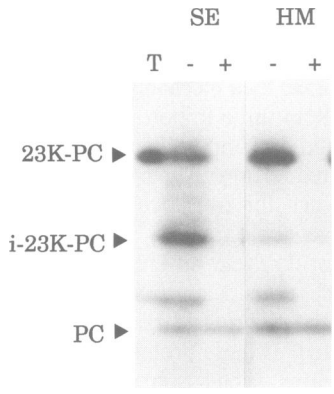

Fig. 3. Stromal extract is not required for the import of a 23K−PC fusion protein into thylakoids. Thylakoid import assays using a fusion protein consisting of the presequence of 23K linked to mature plastocyanin (23K−PC) were carried out in the presence of stromal extract (SE) or in HM buffer. After import, samples were analysed directly or after protease treatment of the thylakoids (−, +, respectively). Lane T, translation product; i23K−PC, intermediate form generated by the stromal processing peptidase activity in the stromal extract.

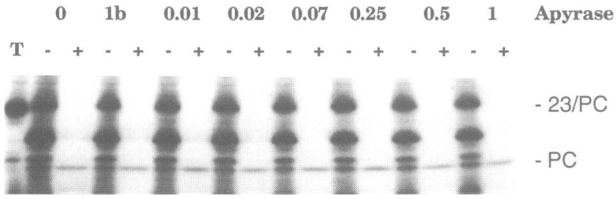

Fig. 4. NTPs are not required for the import of 23K−PC into thylakoids. Thylakoid import assays were carried out using 23K−PC in the presence of stromal extract. Before commencement of the import incubation, import mixtures were treated with apyrase (units indicated above the lanes) as detailed in the legend to Figure 2. The control incubation using boiled apyrase is denoted 1b. Samples were analysed directly or after protease treatment of the thylakoids (−, +). Legends as in Figure 3.

The third analysis of the 23K−PC translocation mechanism is shown in Figure 5, which illustrates the effect of nigericin on the translocation of 23K, plastocyanin and 23K−PC across the thylakoid membrane in intact chloroplasts. In the import assays conducted in the absence of nigericin, all three proteins were efficiently imported into chloroplasts, transported into the thylakoid lumen and processed to the mature size: in each case, the mature size proteins are resistant to protease treatment of the thylakoid fraction (lanes 5). Following import of pre-23K and 23K−PC, some stromal intermediate-size form can be detected, although most of the imported protein is present as the mature form in the thylakoid lumen. Only the mature form can be detected after pre-plastocyanin import, indicating that translocation across the thylakoid membrane proceeds rapidly after import into the chloroplast. As expected from the data of Theg *et al.* (1989), Mould and Robinson (1991) and Cline *et al.* (1992), nigericin has no effect on the import or localization of plastocyanin (panel A) but completely inhibits the transport of 23K across the thylakoid membrane (panel B), with the result that the intermediate form accumulates in the stroma (lanes 3). Figure 5C shows that nigericin also totally inhibits the translocation of 23K−PC across the thylakoid membrane; no mature size form whatsoever can be detected in the protease-treated thylakoid fraction, and the stromal intermediate form again accumulates. Thus, in each of three tests to discriminate between the 23K and plastocyanin translocation mechanisms,

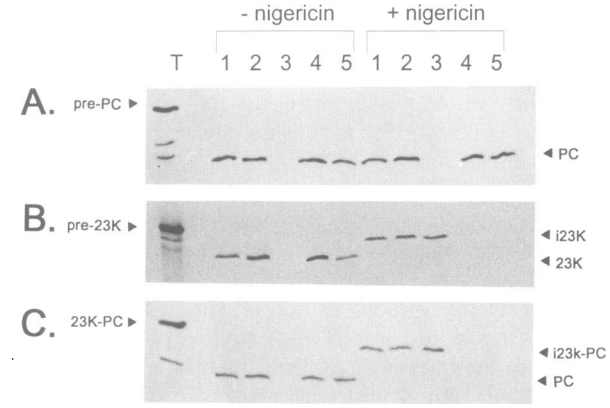

Fig. 5. Effects of nigericin on the import and localization of plastocyanin, 23K and 23K−PC by intact chloroplasts. Isolated intact chloroplasts were preincubated with 8 mM MgATP for 15 min at 25°C, after which methionine was added to 2 mM and pre-plastocyanin (**A**), pre-23K (**B**) or 23K−PC (**C**) translation mixture was added. In the samples shown in the right-hand panels, the incubation mixtures contained 2 μM nigericin, which was added just prior to the translation mixtures from a 125× stock in ethanol. Control incubations (left-hand panels) contained an equivalent concentration of ethanol. Incubation was for 20 min at 25°C, after which samples were diluted with 0.5 ml import buffer, centrifuged in a microfuge for 1 min and the pellets were resuspended in SDS sample buffer (lanes 1). Lanes 2−5: after incubation, thermolysin (0.2 mg/ml) was added and the samples were left on ice for 30 min. Samples were then analysed after pelleting the chloroplasts as above (lanes 2) or after lysing pelleted chloroplasts in 50 μl HM buffer for 10 min on ice, followed by centrifugation in a microfuge for 5 min, after which the stromal supernatant (lanes 3) or membrane pellet (lanes 4) were analysed. Lane 5: thylakoids prepared as in lanes 4 were treated with thermolysin (0.2 mg/ml) for 30 min after which they were diluted with 0.5 ml HM buffer, pelleted and resuspended in SDS sample buffer containing 10 mM EDTA. i23K, i23K−PC: stromal intermediate forms of 23K and 23K−PC, respectively.

the results obtained using the 23K−PC construct are identical to those obtained with 23K and diametrically opposite to the results obtained with plastocyanin.

The above results strongly indicate that 23K and plastocyanin are translocated by separate systems in the thylakoid membrane, with the system responsible for 23K transport specifically recognizing signals in the 23K presequence and thus being capable of transporting 23K−PC entirely by the 23K-type mechanism. Given that 23K and 16K are translocated by very similar mechanisms, it would be expected that a 16K−PC fusion protein would be translocated by a mechanism similar to that of 23K−PC, and this indeed appears to be the case. Unfortunately, this construct is not imported efficiently by isolated thylakoids and so it has not been possible to test whether stromal factors or NTPs are required. However, Figure 6 shows that 16K−PC is imported into the thylakoid lumen of intact chloroplasts, and that as with 23K−PC, the presence of nigericin completely inhibits translocation across the thylakoid membrane. Interestingly, both the intermediate form and the full precursor accumulate in the stroma under these circumstances (lane 4); this construct is evidently a poor substrate for the stromal processing peptidase. As with 23K−PC, therefore, the behaviour of this construct in this assay is dictated exclusively by the origin of the presequence. As yet, however, it has not been possible to determine whether the signals recognized by the other translocation system are localized solely in the plastocyanin presequence, because the reciprocal types of construct (PC−23K and PC−16K) are not imported by isolated thylakoids, and, within intact chloroplasts, are transported either not at all (PC−23K) or at very low rates (PC−16K) across the thylakoid membrane (Clausmeyer *et al.*, 1993; our unpublished data).

Discussion

In this study we have aimed first to examine the mechanism by which plastocyanin is translocated across the thylakoid membrane and secondly to rationalize the notably different translocation mechanisms which have emerged from studies of only a few lumenal proteins. In particular, we have sought to determine whether the observed mechanistic differences are a reflection of (i) the varying characteristics of the mature passenger proteins or (ii) the operation of more than one translocation system in the thylakoid membrane.

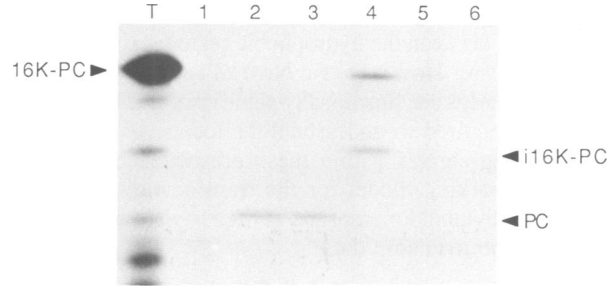

Fig. 6. A delta pH is required for the translocation of a 16K−PC fusion protein across the thylakoid membrane. A 16K−PC fusion protein was incubated with intact pea chloroplasts in the presence (lanes 1−3) and absence (lanes 4−6) of nigericin, under conditions described in the legend to Figure 5. After import, the chloroplasts were treated with protease and samples of the stromal fraction (lanes 1 and 4), thylakoid fraction (lanes 2 and 5) and protease-treated thylakoid fraction (lanes 3 and 6) were analysed. i16K−PC, stromal intermediate form.

Although it has been known for some time that the translocation of plastocyanin across the thylakoid membrane in intact chloroplasts can take place in the absence of a delta pH (Theg *et al.*, 1989), further analysis of the translocation mechanism has proved elusive because attempts to import plastocyanin into isolated thylakoids have met with only limited success. Bauerle and Keegstra (1991) showed that *Arabidopsis* and pea pre-plastocyanin could be imported by pea thylakoids, but with only low efficiency in each case. Fortuitously, and for reasons which are unclear, the spinach precursor protein is imported with efficiencies which permit a more detailed analysis of the translocation mechanism (the improved efficiency is not due to differences in incubation conditions, since we observe only low efficiency import of *S.pratensis* pre-plastocyanin under the same conditions; data not shown). Overall, the translocation mechanism appears to be very similar to the mechanism utilized for the translocation of 33K across the thylakoid membrane, since the presence of a stromal factor(s) and NTPs are critical elements of both mechanisms. In fact, the mechanisms may well be identical. In view of the above similarities, we have re-examined the effects of nigericin on the translocation of 33K across the thylakoid membrane and we have observed only a slight inhibition of translocation in intact chloroplasts (data not shown). Cline *et al.* (1992) similarly found that nigericin had only a minor effect on the translocation of pea 33K across the thylakoid membrane. At present, we have no explanation for the more drastic effects of nigericin which were observed by Mould and Robinson (1991), and we are currently testing the effects of other uncouplers on 33K translocation in an attempt to resolve finally this question. One possibility under consideration is that a delta pH plays a more significant role in the translocation of 33K (and possibly plastocyanin) when ATP concentrations in the chloroplast are relatively low. Whatever the explanation, it now appears likely that both plastocyanin and 33K are translocated across the thylakoid membrane by a mechanism which does not require a delta pH, although a delta pH may stimulate translocation under some circumstances. If this is indeed the case, the four lumenal proteins studied in detail to date can be divided into two groups on the basis of their translocation mechanisms. 23K and 16K are transported by a mechanism which appears only to require a transthylakoidal delta pH, with no evidence for any role of soluble stromal factors or NTP hydrolysis in the translocation process. In contrast, 33K and plastocyanin are transported by a more complex mechanism which requires the presence of at least one stromal factor and NTPs, but for which a delta pH is not a prerequisite.

Further work is required to determine the nature and role of the stromal factor involved in plastocyanin translocation, since this factor has yet to be characterized in any detail. This was not one of the aims of this study; our primary objective was to test the influences of the presequence and mature sequence on 23K and plastocyanin translocation mechanisms, and irrespective of the nature of the stromal factor, it is clearly essential for efficient plastocyanin translocation but completely unnecessary for translocation of 23K (or 16K). However, it is of course possible that different stromal factors are required for the translocation of 33K and plastocyanin, and this possibility will be addressed in future studies.

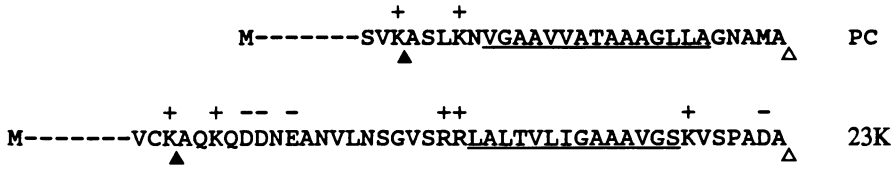

Fig. 7. Structures of the 23K and plastocyanin thylakoid transfer signals. The structures of the thylakoid transfer signals of spinach 23K and plastocyanin (denoted PC) are shown. The signals follow the intermediate SPP cleavage sites (filled arrows) and extend until the terminal TPP cleavage sites (open arrows). The SPP cleavage site in spinach pre-23K has been deduced by Edman degradation (our unpublished data) and found to coincide with the site deduced for wheat pre-23K by Bassham *et al.* (1991). The SPP cleavage site for spinach pre-plastocyanin is assumed to be at the site corresponding to that identified within *Silene* pre-plastocyanin by Bassham *et al.* (1991), since the two species of precursor contain very similar transfer signals. Both signals contain apparently similar hydrophobic core sequences (underlined) and signal peptidase-type cleavage sites ending with the AXA consensus motif.

It is useful that the translocation mechanism for 33K and plastocyanin differs so radically from that for 23K and 16K in three respects, because interpretation of the data using the 23K−PC fusion protein is made much easier. The data clearly indicate that the translocation of this protein across the thylakoid membrane is absolutely dependent on a delta pH, but does not require stromal factors or NTPs; in other words, the behaviour of this protein under these conditions is indistinguishable from that of the precursor from which the presequence was derived: pre-23K. Given these findings, we conclude that the stromal factor and NTPs required for the transport of pre-plastocyanin are not involved in somehow rendering the plastocyanin mature protein more translocation-competent. The data also preclude the possibility that 23K and plastocyanin are transported by a single translocator, with the characteristics of the plastocyanin mature protein somehow facilitating translocation in the absence of a delta pH; when fused behind the 23K presequence, translocation of plastocyanin is totally dependent on a delta pH. It is theoretically possible that the stromal factor and NTPs are specifically required for the translocation of the plastocyanin thylakoid transfer signal across the thylakoid membrane (and by the same token, particular characteristics of the 23K and 16K transfer signals may demand that a delta pH is harnessed to drive translocation of the signal across the thylakoid membrane). However, the available evidence argues against these possibilities, primarily because the two types of translocation mechanism differ completely in three fundamental respects, but also because the thylakoid transfer signals of these proteins are far more similar than are the mature proteins. Instead, the most probable explanation of these data is that 23K and plastocyanin are transported by separate translocases in the thylakoid membrane and that the 23K system recognizes specific signals in the presequence (presumably within the thylakoid transfer signal) of a subset of lumenal proteins, and is able to quantitatively divert 'foreign' proteins (such as plastocyanin) if preceded by a 23K presequence. If this is the case, the translocation requirements of 33K and 16K, and the import characteristics of the 16K−PC fusion protein, are such that it is likely that 23K and 16K are transported by one translocase, and 33K and plastocyanin by another. It is as yet unclear whether the putative plastocyanin/33K system recognizes signals which are restricted to the thylakoid transfer signals of these proteins.

The existence of two such different translocation mechanisms is unexpected because the thylakoid transfer signals of 23K and plastocyanin, which are detailed in Figure 7, share at least two features in common. Both signals contain hydrophobic core sections (underlined) of similar length and moment, together with a signal peptidase-type processing signal (AXA) at the carboxy-terminus. It therefore appears likely that the critical differences in these signals lie in the amino-terminal regions, which are indeed different within these proteins: the 23K signal is both longer and more highly charged and the two signals also differ in terms of the distance between the hydrophobic region and the terminal processing site. However, we have as yet no evidence that these differences are functionally significant and further work is clearly required to understand the location and forms of the targeting signals within these presequences. Figure 8 depicts a working model for the translocation of proteins across the thylakoid membrane in higher plant chloroplasts, based on the available data.

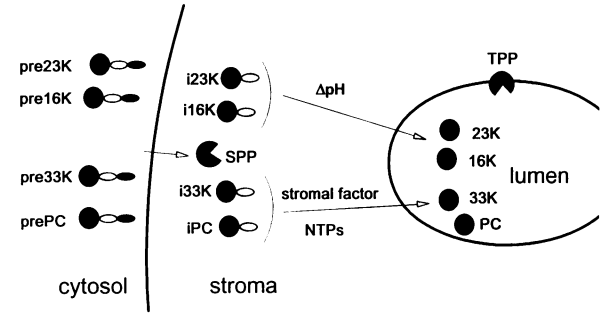

Fig. 8. Working model depicting the mechanisms by which lumenal protein precursors are imported and sorted by higher plant chloroplasts. Precursors of the four lumenal proteins are synthesized in the cytosol with bipartite presequences containing an envelope transit (black ovals) and thylakoid transfer signal (shaded ovals) in tandem. The precursors are imported into the stroma, probably by a common mechanism, and are processed to intermediate forms by an SPP. The thylakoid transfer signals then direct translocation across the thylakoid membrane by two distinct mechanisms, probably mediated by two distinct translocases in the thylakoid membrane. Translocation of 23K and 16K requires only a delta pH across the thylakoid membrane, whereas translocation of 33K and plastocyanin requires at least one stromal factor and NTPs (probably ATP), but can take place in the absence of a delta pH. After translocation is carried out, all four proteins are probably processed to the mature size by a common thylakoidal processing peptidase (TPP).

Materials and methods

Plants

Seedlings of *Pisum sativum*, var. Feltham first, were grown for 8−9 days under a 12 h photoperiod. Light intensity was 50 μmol photons/m/s.

Generation of precursor proteins

Precursors were synthesized by transcription *in vitro* of cDNA clones, followed by translation in a wheat germ lysate in the presence of [^{35}S]methionine. The synthesis of wheat pre-23K has been described

previously (James *et al.*, 1989). A spinach genomic clone encoding preplastocyanin (Rother *et al.*, 1986) was cloned into pBluescript (Stratagene) and transcribed using T3 RNA polymerase. The construction of the 23K–PC and 16K–PC chimeric clones is detailed in Clausmeyer *et al.* (1993).

Thylakoid import assays

Intact pea chloroplasts isolated as described (Hageman *et al.*, 1986) were pelleted and lysed in 10 mM HEPES–KOH, pH 8.0, 5 mM $MgCl_2$ (HM buffer) to a final concentration of 0.75 mg/ml chlorophyll. After incubation on ice for 10 min, the thylakoids were pelleted in a microfuge (10 000 r.p.m. for 5 min) and the stromal supernatant was kept if required. The thylakoids were washed twice in HM buffer and finally resuspended in either HM or the stromal fraction to a concentration of 0.75 mg/ml chlorophyll. Import incubations contained 45 μl thylakoid suspension and 5 μl translation mixture containing precursor. All thylakoid isolation steps were carried out at 4°C and pea thylakoids were incubated with precursor no more than 60 min after the onset of the isolation procedure and the *in vitro* translation reaction. Incubation was for 20 min at 25°C under illumination (150 μmol photons/m/s), after which half of the mixture was mixed with SDS sample buffer and boiled for 2 min. The remaining 25 μl were diluted with 0.5 ml HM buffer and the thylakoids were pelleted in a microfuge; the thylakoid pellet was resuspended in 25 μl HM and incubated with thermolysin (0.2 mg/ml) for 30 min on ice. After incubation, the sample was diluted with 0.5 ml HM buffer containing 10 mM EDTA and the thylakoids were pelleted and boiled in SDS sample buffer. Samples were analysed by SDS–PAGE and fluorography. Import was quantified by scanning autoradiographs using a Molecular Dynamics Imagequant laser densitometer v. 3.0.

Chloroplast import assays

Chloroplast import assays and subsequent fractionations were carried out as described by Mould and Robinson (1991), except that nigericin, when present, was used at a final concentration of 2 μM. The nigericin was added from a 125× stock solution in ethanol; control import incubations contained an equivalent concentration of ethanol.

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