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# Transport of proteins into chloroplasts

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All cells transport proteins across membranes, but the complexity of protein traffic in plant cells is especially striking because of the variety of organelle types involved. Many proteins are inserted, during translation, into the lumen of the endoplasmic reticulum, after which they are transported via the endomembrane system to the Golgi apparatus, vacuole, protein bodies or plasma membrane. Other proteins are transported post-translationally into glyoxysomes, mitochondria and plastids. In each case, the protein is synthesised with an appropriate signal which ensures targeting to the correct organelle, and a number of studies have attempted to define the characteristics of these targeting signals (reviewed by Bennett & Osteryoung, 1991; Robinson, 1991).

In terms of protein transport events, the biogenesis of the chloroplast is particularly complex, primarily owing to the architecture of the organelle. The chloroplast is bounded by a double-membrane envelope, between whose membranes is a soluble phase, the functions of which are presently obscure. Within the organelle is the soluble stromal phase (site of $CO_2$ fixation, amino acid synthesis and many other key reactions) and the extensive internal thylakoid membrane. The thylakoid network also encloses a further soluble phase, usually termed the thylakoid lumen. Thus, the chloroplast comprises in total three distinct membranes and three discrete soluble phases. Most of the proteins located in each of these organellar compartments are encoded by nuclear genes, synthesised in the cytosol, and transported into the organelle. Clearly, therefore, chloroplast biogenesis requires both the specific, efficient targeting of a large number proteins into the organelle, and the operation of intraorganellar 'sorting' mechanisms to distribute imported proteins to their correct destinations. Many of the underlying mechanisms are at present poorly understood, but substantial advances have been made in recent years in studies on the biogenesis of thylakoid proteins. In this chapter we will consider three key aspects of the import and sorting of thylakoid lumen proteins: the targeting signals involved, the mechanism

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of removal of these signals, and the energetics of protein translocation across the thylakoid membrane.

## Biogenesis of thylakoid lumen proteins

Most of the abundant thylakoid proteins are components of the four protein complexes that account for the bulk of thylakoid protein: photosystem I, photosystem II, the cytochrome *b/f* complex, and the ATP synthetase complex. Many of these proteins are synthesised in the cytosol, and these proteins must therefore cross the envelope membranes and the stromal phase to reach the thylakoid membrane. Of particular interest is the biogenesis of hydrophilic thylakoid lumen proteins, since these must cross all three chloroplast membranes to reach their sites of function. The best-studied lumenal proteins are plastocyanin, a small copper-containing electron carrier, and three extrinsic photosystem II proteins of 33, 23 and 16 kDa which are components of the oxygen-evolving complex (Murato & Miyao, 1985; Andersson, 1986). The locations of these three proteins (33K, 23K and 16K) are shown diagrammatically in Fig. 1.

The import pathway taken by these proteins can be divided into two phases (Fig. 2). Initially, the proteins are synthesised in the cytosol as larger precursors containing amino-terminal precursors, transported into the stroma, and cleaved to intermediate-sized forms by a stromal processing peptidase (SPP). Thereafter, the intermediates are transported

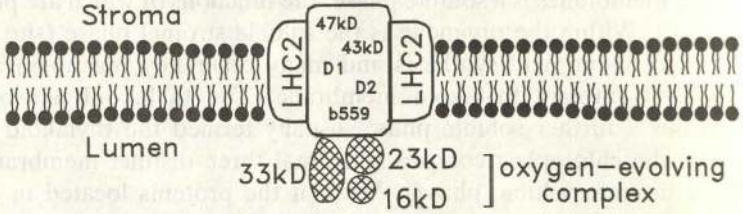


Fig. 1. Simplified diagram of the Photosystem II complex. The PSII complex contains a reaction centre core complex, which consists of the D1 and D2 proteins, cytochrome *b*559, and polypeptides of 43 and 47 kDa; a light-harvesting protein-pigment network of which the major protein component is the light-harvesting chlorophyll-binding protein (LHC-2); and a peripheral oxygen-evolving complex. The major components of the oxygen-evolving complex are three extrinisic polypeptides of 33, 23 and 16 kDa; these polypeptides and LHC-2 are nuclear-encoded and imported from the cytosol whereas the core complex polypeptides are chloroplast-encoded.

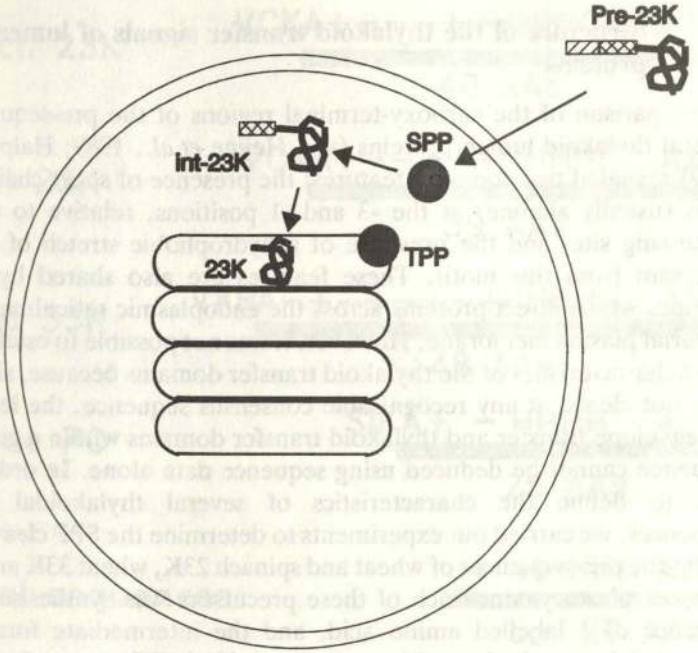


Fig. 2. Two-step model for the import of thylakoid lumen proteins. Lumenal proteins such as the 23 kDa protein of the oxygen-evolving complex (23K) are synthesised in the cytosol with a bipartite pre-sequence. After synthesis, pre-23K is imported into the stroma and cleaved to an intermediate form (int-23K) by a stromal processing peptidase (SPP). Int-23K is subsequently transported across the thylakoid membrane and processed to the mature size by a thylakoidal processing peptidase, TPP.

across the thylakoid membrane and processed to the mature sizes by a second, thylakoidal processing peptidase TPP (Hageman *et al.*, 1986; Smeekens *et al.*, 1986; James *et al.*, 1989). In keeping with this import mechanism, the pre-sequences of lumenal proteins contain two distinct domains which have differing characteristics. The amino-terminal 'envelope transfer' domains are structurally and functionally equivalent to the pre-sequences of imported stromal proteins, being rich in positively charged and hydroxylated residues. The second, 'thylakoid transfer' sequences have markedly different features which are discussed in the following section.

## Structure of the thylakoid transfer signals of lumenal proteins

A comparison of the carboxy-terminal regions of the pre-sequences of several thylakoid lumen proteins (von Heijne *et al.*, 1989; Halpin *et al.*, 1989) revealed two common features: the presence of short-chain amino acids (usually alanine) at the -3 and -1 positions, relative to the TPP processing site, and the presence of a hydrophobic stretch of residues upstream from this motif. These features are also shared by 'signal' peptides which direct proteins across the endoplasmic reticulum and the bacterial plasma membrane. However, it was not possible to establish any other characteristics of the thylakoid transfer domains because, since SPP does not cleave at any recognisable consensus sequence, the lengths of the envelope transfer and thylakoid transfer domains within a given pre-sequence cannot be deduced using sequence data alone. In order to be able to define the characteristics of several thylakoidal transfer sequences, we carried out experiments to determine the SPP cleavage site within the pre-sequences of wheat and spinach 23K, wheat 33K and *Silene pratensis* plastocyanin. Each of these precursors was synthesised in the presence of a labelled amino acid, and the intermediate forms were generated by incubation with partially purified SPP as previously described (Hageman *et al.*, 1986; James *et al.*, 1989). The intermediates were then subjected to automated Edman degradation, and the SPP cleavage sites were deduced from the cycle numbers at which the radiolabelled amino acids were released. Figure 3 shows some of the features of the thylakoid transfer signals, and compares these features with those of typical signal sequences, after analysis of the thylakoid transfer sequences within the total pre-sequence (Smeekens *et al.*, 1985; Meadows *et al.*, 1991).

One of the most surprising findings to emerge from this study concerned the length of these targeting signals. Signal peptides are typically about 20 residues in length, but the 23K and 33K thylakoid transfer domains are at least twice as long as this. The corresponding sequence from plastocyanin is, however, more similar to signal peptides in terms of length, but this sequence is probably atypically short since the pre-sequence as a whole is unusually small.

The 23K and 33K transfer sequences are also markedly different in terms of charge distribution from typical signal sequences. An important feature in both prokaryotic and eukaryotic signal peptides is the presence of one or two positive charges between the amino terminus and the hydrophobic section (von Heijne, 1986). In contrast, this sequence in the 23K and 33K thylakoid transfer domains is much longer and contains

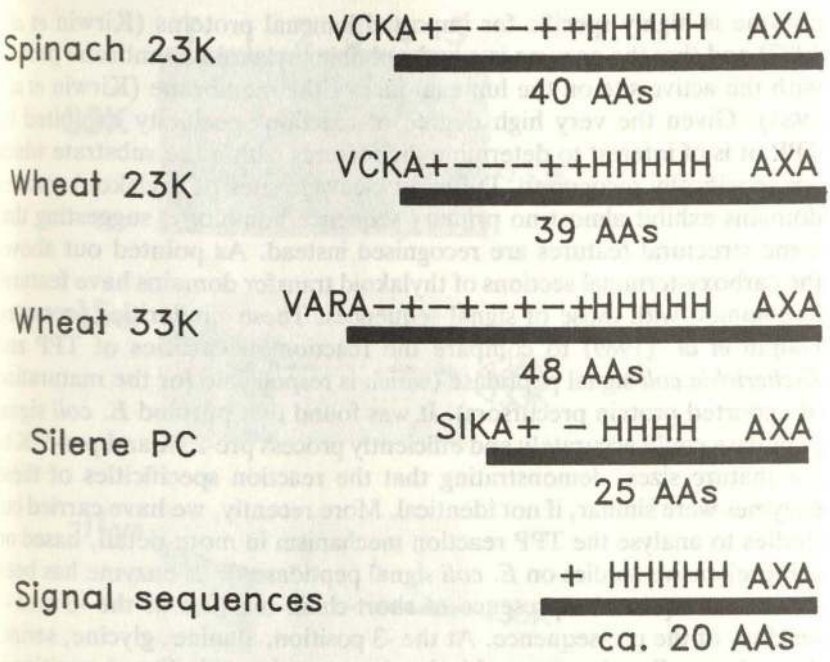


Fig. 3. Structural features of thylakoid transfer sequences. The carboxy-terminal regions of thylakoid transfer domains resemble signal sequences in two respects: the presence of a stretch of hydrophobic residues (H) and the presence of short-chain residues, usually alanine, at the -3 and -1 positions. The two types of peptide can have markedly different amino-terminal sections, especially in the cases of the 33K and 23K sequences, where the transfer sequences are much longer than signal sequences, and contain numerous negatively and positively charged residues. Signal sequences usually contain one or two positive charges in this region.

numerous positive *and* negative charges in equal numbers. The functional significance of this charged region is presently unclear, but the presence of this feature means that these thylakoid transfer domains are, in overall terms, similar to signal sequences in some respects but also dissimilar in others.

## Mechanism of the thylakoidal processing peptidase

During or shortly after translocation of import intermediates across the thylakoid membrane, the thylakoid transfer sequence is removed by TPP to release the mature-size protein. Studies on TPP have shown that this

enzyme is highly specific for imported lumenal proteins (Kirwin *et al.*, 1987) and that the enzyme is a hydrophobic thylakoid membrane protein with the active site on the lumenal face of the membrane (Kirwin *et al.*, 1988). Given the very high degree of reaction specificity exhibited by TPP, it is of interest to determine the features within the substrate which are specifically recognised. Different cleavage sites of thylakoid transfer domains exhibit almost no primary sequence homology, suggesting that some structural features are recognised instead. As pointed out above, the carboxy-terminal sections of thylakoid transfer domains have features in common with those of signal sequences. These similarities prompted Halpin *et al.* (1989) to compare the reaction specificities of TPP and *Escherichia coli* signal peptidase (which is responsible for the maturation of exported protein precursors). It was found that purified *E. coli* signal peptidase could accurately and efficiently process pre-23K and pre-33K to the mature sizes, demonstrating that the reaction specificities of these enzymes were similar, if not identical. More recently, we have carried out studies to analyse the TPP reaction mechanism in more detail, based on comprehensive studies on *E. coli* signal peptidase. This enzyme has been shown to require the presence of short-chain residues at the -3 and -1 residues of the pre-sequence. At the -3 position, alanine, glycine, serine, threonine, valine, leucine and isoleucine are tolerated. The -1 position is more restrictive, and only alanine, serine, glycine and cysteine are tolerated (von Heijne, 1986; Folz *et al.*, 1988; Fikes *et al.*, 1990).

To test the requirements for cleavage by TPP, we used site-specific mutagenesis to substitute the -3 and -1 residues of wheat pre-33K (both of which are alanine) by a variety of amino acids. The mutant precursors were then expressed by *in vitro* transcription–translation and imported into intact chloroplasts to monitor the effects of the mutations on membrane translocation and maturation. Figure 4 shows the results of import assays using wild-type pre-33K and two of the mutants, -1 Ser and -1 Lys. Wild-type pre-33K is imported into the thylakoids where it is resistant to added protease, showing that the protein is located in the lumen. Both of the mutants are also transported into the lumen, and studies (not shown) on the remaining mutants give a similar result: none of the -3 or -1 mutations affect transport across either the envelope or the thylakoid membranes. However, in almost every case, cleavage by TPP is dramatically affected. In the case of -1 Ser, very little of the imported protein is processed to the mature size, and the majority is instead processed only to a 36 kDa polypeptide (36K). This polypeptide is slightly smaller than the stromal intermediate generated by SPP, and our interpretation is that TPP, being unable to cleave efficiently at the correct processing site as a result of the mutation, is recognising a cryptic processing site elsewhere in

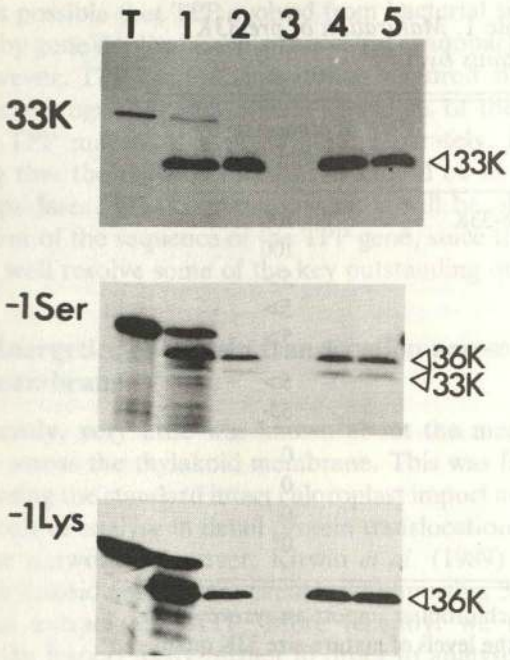


Fig. 4. Import of mutated pre-33Ks into isolated chloroplasts. The pre-33K cDNA was subjected to site-specific mutagenesis in order to substitute a variety of amino acids for the -3 and -1 alanine residues of the pre-sequence. The autoradiogram shows the import and localisation of wild-type pre-33K and two of the mutants: -1 Ser and -1 Lys. Lanes T, translation product; lanes 1, total polypeptides after import; lanes 2, after protease treatment of the chloroplasts; lanes 3 and 4, after fractionation into stroma and thylakoids, respectively; lanes 5, after protease treatment of the thylakoids. 33K: mobility of mature 33K; 36K: 36kDa cleavage product.

the thylakoid transfer sequence (Shackleton & Robinson, 1991). In the case of imported -1 Lys mutant, only the 36K polypeptide is detected in the thylakoid fraction indicating that this mutation completely blocks cleavage by TPP at the correct site.

Time course analyses of the maturation of imported protein were carried out for all of the mutants, and the results are summarised in Table 1. Of the 10 mutants tested, only one (-3 Val) is efficiently processed by TPP at the correct site. The other -3 mutations (-3 Leu, Glu and Lys) all drastically inhibit cleavage, leading to the generation of mostly 36K polypeptide. With the -1 mutations, four of the substitutions completely

Table 1. *Maturation of pre-33K mutants by TPP*

| Construct | Rate of processing by TPP (% of wild-type rate) |
|---|---|
| Wild-type pre-33K | 100 |
| -3 Val | 100 |
| -3 Leu | 5> |
| -3 Lys | 5> |
| -3 Glu | 5> |
| -1 Ser | 5> |
| -1 Gly | 5> |
| -1 Thr | 0 |
| -1 Glu | 0 |
| -1 Lys | 0 |
| -1 Leu | 0 |

Time-course chloroplast import assays were carried out and the levels of mature-size 33K quantitated by laser densitometry. In all cases, inhibition of cleavage at the correct site by TPP resulted in accumulation of a 36 kDa intermediate, as described in Fig. 4.

block cleavage by TPP at the correct site (-1 Thr, Leu, Glu and Lys). Of particular interest, however, the -1 Gly and -1 Ser substitutions also drastically inhibit cleavage, even though these residues are very similar in overall structure to the -1 Ala in the wild-type precursor.

The conclusion from this study is that, although TPP appears to be similar in terms of overall reaction mechanism to signal peptidases, TPP has far more stringent requirements at the -3 and -1 residues of the substrate than either bacterial or ER signal peptidases. Whereas the latter enzymes can tolerate leucine at the -3 position, the presence of this residue almost completely blocks cleavage by TPP. It appears likely that the valine side-chain represents the maximum permitted length at this position. At the -1 position, TPP has very strict requirements in that alanine appears to be essential for efficient cleavage to take place. Even glycine and serine, which are commonly found at the -1 position in bacterial and eukaryotic signal peptides, are not tolerated.

These results raise interesting possibilities concerning the evolution of

TPP. It is possible that TPP evolved from bacterial signal peptidase, for example by gene duplication in an ancestral cyanobacterium; if this is the case, however, TPP has for some reason acquired much more stringent requirements regarding the -3 and -1 residues of the substrates. Alternatively, TPP may have evolved quite separately, in which case it is intriguing that the reaction mechanism should be so similar to those of signal peptidases. Whatever the reason, it will be of interest to obtain information of the sequence of the TPP gene, since this type of information may well resolve some of the key outstanding questions.

## Energetics of protein translocation across the thylakoid membrane

Until recently, very little was known about the mechanism of protein transport across the thylakoid membrane. This was for largely technical reasons: using the standard intact chloroplast import assay, it is in practise very difficult to analyse in detail protein translocation across the internal membrane network. However, Kirwin *et al.* (1989) demonstrated that isolated thylakoid vesicles are capable of importing 33K in the presence of stromal extract and ATP. More recently, we have attempted to optimise the import assay further in order to analyse the mechanism of the transport system. We found that a critical requirement for efficient import of both 23K and 33K is the presence of light (Mould & Robinson, 1991). It seemed likely that this reflected a role, either directly or indirectly, of thylakoidal electron transport in promoting protein translocation, and experiments were carried out to test this possibility. Import assays were carried out in the presence of electron transport inhibitors (a combination of dichlorophenyl-dimethylurea (DCMU) and methyl viologen) to block formation of the thylakoidal protonmotive force ($\Delta p$, also known as PMF). Other assays were carried out in the presence of valinomycin (which selectively dissipates the electrical potential component, $\Delta\psi$, of the $\Delta p$) and nigericin, which collapses the proton gradient component, $\Delta pH$. Figure 5 shows the result of a thylakoid import assay using pre-23K as a substrate. In the presence of stroma and light, pre-23K is converted to the intermediate form (by SPP in the stromal extract) and also to the mature size (lane 1). When this mixture is protease-treated after incubation (lane 5) the mature-size 23K is protected from digestion, showing that these molecules have been imported into the lumen. In the presence of DCMU/methyl viologen, pre-23K is efficiently processed to the intermediate form but no mature-size polypeptide appears, showing that import has been completely inhibited (lanes 2 and 6). The presence of valinomycin (lanes 3 and 7) leads to a very slight inhibition of import,

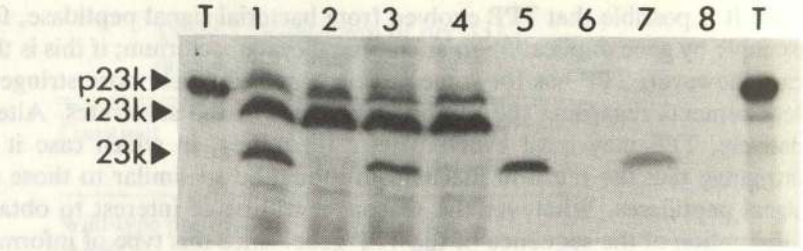


Fig. 5. Light-dependent import of 23K by isolated thylakoids. Pre-23K was incubated with stroma and thylakoids in the light (lane 1), and in the presence of DCMU/methy viologen (lane 2), valinomycin (lane 3) or nigericin (lane 4). Lanes 5–8, as in lanes 1–4 except that the thyalkoids were protease-treated after incubation. Lane T, translation product; p23K, i23K, precursor and intermediate forms of 23K.

but nigericin again blocks import completely (lanes 4 and 8). We conclude from this experiment that protein transport across the thylakoid membranes requires an energised membrane, and that the dominant component driving the translocation process is the proton gradient, and not the electrical potential, of the total protonmotive force. It remains to be determined precisely how $\Delta p$ drives protein transport across the thylakoid membrane.

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