Novel mechanisms for the targeting of proteins into and across chloroplast membranes
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Abstract
The assembly of the photosynthetic apparatus requires the translocation of numerous proteins from the cytosol, initially into the stroma and thereafter into or across the thylakoid membrane. Recent studies have shown that proteins are transported into this membrane by a variety of mechanisms, some of which are derived from a cyanobacterial-type ancestor, whereas others have evolved in response to the more complex transport pathway used by cytosolically synthesized chloroplast proteins. It is now apparent that some of the targeting pathways are used exclusively by hydrophobic thylakoid membrane proteins; here we review recent progress in our understanding of the biogenesis of this important class of protein.

Introduction
Membrane proteins perform an enormous array of functions and their modes of synthesis have attracted a great deal of interest. These hydrophobic proteins must avoid aggregation during and after synthesis, after which they must be efficiently transferred into the correct lipid bilayer. Most importantly, they must assume the correct topology during the insertion process, because there are very few examples of membrane-spanning regions reversing topology once inserted.

The chloroplast thylakoid membrane has been a popular model system for the analysis of membrane protein biogenesis because this membrane performs the crucial processes of light capture, photosynthetic electron transport and ATP synthesis. Many of the component proteins are very abundant indeed and several have been studied in some detail. In addition, however, the biogenesis of membrane proteins in chloroplasts is made more complex (and hence perhaps more interesting) because of the architecture of the organelle and the dual genetic origins of chloroplast proteins. The chloroplast contains its own genome and synthesizes a number of proteins: approximately 80 in higher plants, of which many are thylakoid membrane proteins. The remaining thylakoid proteins are synthesized in the cytosol and must therefore be imported across the double-membrane envelope and soluble stromal phase before they can be inserted into the thylakoid membrane. Finally, it should be borne in mind that other hydrophobic proteins must be targeted into the envelope membranes rather than the thylakoid membrane. Thus this combination of factors means that effective targeting pathways must exist for thylakoid membrane proteins, especially those that originate from the cytosol. Here we discuss the known targeting pathways for nuclear-encoded thylakoid membrane proteins; additional pathways for lumen proteins will not be considered but are reviewed elsewhere [1,2].

Signal recognition particle (SRP)-dependent insertion pathway
The major light-harvesting chlorophyll-binding protein, Lhcb1, is the most intensively studied
thylakoid membrane protein; much of our current knowledge is based on studies of this three-membrane-span protein. Lhcb1 is synthesized in the cytosol with an N-terminal presequence that functions to target the protein into the stroma, apparently using a general mechanism for transport across the envelope membranes. The presequence can be replaced with that of a stromal protein without affecting thylakoid targeting [3,4]. The insertion of Lhcb1 into the thylakoid membrane has been reconstituted in vitro in assays where radioactively labelled protein, prepared in a cell-free translation system, is mixed with isolated thylakoid vesicles. This type of study has shown that Lhcb1 binds to a stromal form of SRP, apparently to form a soluble transit complex en route to the thylakoids [5,6]. A second soluble factor, FtsY, is also involved in the insertion mechanism [7] and the overall insertion process has been shown to depend on GTP hydrolysis [8].

This seems to be a prime example of evolutionary conservation in targeting pathways because the same factors have been found to be essential for the insertion of a range of bacterial inner-membrane proteins (reviewed in [1,9,10]). SRP is a GTP-binding protein that binds preferentially to highly hydrophobic regions, after which FtsY, another GTP-binding protein, somehow transfers the membrane protein from SRP into translocation machinery in the bacterial membrane. Cross-linking studies have suggested that FtsY acts as form of adaptor, using energy derived from GTP hydrolysis to insert the membrane protein substrate partly into the membrane [11]. There are, however, some differences between the bacterial and chloroplastic SRP-dependent pathways. In bacteria, SRP comprises a 48 kDa protein together with a 4.5 S RNA molecule, whereas the chloroplastic particle consists of a 54 kDa homologue of the 48 kDa protein together with a novel 43 kDa protein [12]. Possibly, this difference might reflect the fact that in chloroplasts the soluble part of the targeting pathway is more protracted and the SRP molecule might therefore need to act rather differently.

In Escherichia coli, the membrane-bound SecYEG translocon has been implicated as being important in the later stages of the insertion process [11,13]. Because this translocon is also used for the translocation of soluble proteins into the periplasm, it seems reasonable that it might function similarly in the insertion of membrane proteins. However, it should be emphasized that many of the relevant studies have been performed under Sec-depletion conditions and the actual role of the Sec machinery in bacterial membrane protein biogenesis is unclear at present. Mild proteolysis of thylakoids abolishes their ability to import substrates by the SRP-dependent route [14], demonstrating that some form of translocation apparatus is similarly essential. However, the identity of this apparatus and the overall insertion mechanism are again unclear; recent studies have raised important questions about the

Figure 1

Model for the insertion of thylakoid membrane proteins by the SRP-dependent pathway

After import into the chloroplast, Lhcb1 is believed to interact strongly with SRP, a complex of 54 kDa and 43 kDa subunits, which mediates its delivery to the thylakoid membrane. At this point the transit complex interacts with FtsY, which assists in the membrane-insertion process during which both SRP and FtsY probably use energy derived from GTP hydrolysis to drive substrate release and/or insertion into the membrane. Albino3 (Alb3) is closely involved in the insertion event, possibly as the initial receptor in the membrane. By analogy with bacterial systems, the SecYEG translocon is probably also involved in insertion, although this remains to be confirmed.
membrane-insertion process. Antibodies raised against SecY are able to block the Sec-dependent transport of thylakoid lumen proteins but have no effect on the SRP-dependent insertion of Lhcb1 [15]. However, antibodies raised against another thylakoid membrane protein, Albino3, do block the insertion of Lhcb1 very effectively [16]. This is an interesting finding because Albino3 is related to a mitochondrial protein, Oxa1, that is involved in the insertion of several proteins from the matrix into the mitochondrial inner membrane [17]. Taken together, these results suggest that Oxa1/Albino3-type proteins might represent a new form of translocation apparatus dedicated to the insertion of hydrophobic proteins. It remains to be determined whether, in thylakoids, Albino3 interacts with the SecYEG complex during the final stages of the insertion pathway. Figure 1 illustrates a possible insertion pathway for SRP substrates such as Lhcb1. In this pathway, SRP probably functions both as a targeting factor and an anti-folding chaperone, serving to maintain the protein in a soluble form in the stroma.

**Direct insertion pathway for thylakoid membrane proteins**

Similar types of insertion assay *in vitro* have been used to study other thylakoid membrane proteins, with surprising results. A series of small single-span proteins, namely CF11, PsbW and PsbX, are synthesized in the cytosol as larger precursors but in these cases the presequence is bipartite in structure and strongly resembles those of Sec-dependent luminal proteins. This initially suggested that, after entry into the chloroplast, the hydrophobic signal peptides would interact with the Sec machinery. However, these proteins insert into thylakoid membranes in the complete absence of stromal factors or nucleoside triphosphates; treatment of thylakoids with protease, which effectively destroys the Sec machinery, has no effect on the insertion of these proteins [14,18,19]. These findings prompted comparisons with M13 procoat, the precursor of the phage coat protein, which is similarly synthesized with a signal peptide in *E. coli* and is known to insert into the plasma membrane in an SRP/Sec-independent manner [20]. Because none of the known translocation machinery is involved, these proteins might well insert spontaneously into the thylakoid membrane, in which case the overall insertion mechanism must differ in fundamental respects from that used by the SRP-dependent pathway. However, it should be stressed that true spontaneous insertion will only be proved when this insertion process has been shown to occur with liposomes rather than thylakoid membranes; it remains possible that unknown (and trypsin-resistant) factors might aid insertion.

In the light of the above findings, it has been proposed that the role of the signal peptide in these precursor proteins is not to interact with proteinaceous translocation machinery but is instead to provide an additional hydrophobic region that, in concert with that in the mature protein, inserts into the bilayer with sufficient force to drive the intervening region across the membrane. Thereafter, cleavage on the *trans* side of the membrane by the signal peptidase releases the mature protein. Consistent with this model are studies that have shown that M13 procoat and pre-PsbW both adopt loop structures before cleavage in which the N-
terminus and C-terminus are exposed on the cis side of the membrane [21,22]. This pathway for insertion is illustrated in Figure 2. However, it should be emphasized that, although the protein is shown as inserting directly into the thylakoid membrane, the possibility cannot be ruled out that a membrane-bound apparatus has a role in the insertion process. One clear possibility, for example, is that Albino3 might have a role.

This simpler insertion process was initially thought to be restricted to simple single-span proteins such as PsbW; however, more recent findings have shown that this SRP/Sec-independent insertion process (or variants thereof) are also used by much more complex proteins. One of the most intriguing is PsbY. This protein is synthesized in the cytosol as an approx. 21 kDa precursor protein, imported into the chloroplast and then targeted to the thylakoid membrane. Uniquely, this protein then inserts as a double-loop structure with the use of two distinct signal peptides, each of which precedes a single-span 'mature' protein. This polypeptide is then cleaved twice by the thylakoidal signal peptidase, after which an unknown protease cleaves the remaining junction on the stromal side of the membrane. Remarkably, this complex protein is able to insert with high efficiency in the complete absence of SRP, nucleoside triphosphates or the Sec apparatus [23].

Concluding remarks

Much is now known about the insertion of thylakoid membrane proteins in terms of the factors involved, the energy sources required and the overall insertion pathways. Clearly, many key questions remain but the advent of efficient insertion assays, and the identification of some of the more important targeting factors, mean that the central questions can now be addressed with some confidence. In some respects the thylakoid system is ideal for a detailed dissection of the insertion processes, primarily because proteins synthesized in vitro insert so efficiently into isolated membranes. This experimental advantage should be of enormous help in efforts to understand finally how hydrophobic proteins are inserted into membranes so efficiently and so accurately.

References


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