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Review

Molecular chaperone involvement in chloroplast protein import ☆

Úrsula Flores-Pérez, Paul Jarvis *

Department of Biology, University of Leicester, Leicester LE1 7RH, UK

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ABSTRACT

Chloroplasts are organelles of endosymbiotic origin that perform essential functions in plants. They contain about 3000 different proteins, the vast majority of which are nucleus-encoded, synthesized in precursor form in the cytosol, and transported into the chloroplasts post-translationally. These preproteins are generally imported via envelope complexes termed TOC and TIC (*T*ranslocon at the *O*uter/*I*nner envelope membrane of *C*hloroplasts). They must navigate different cellular and organellar compartments (e.g., the cytosol, the outer and inner envelope membranes, the intermembrane space, and the stroma) before arriving at their final destination. It is generally considered that preproteins are imported in a largely unfolded state, and the whole process is energy-dependent. Several chaperones and cochaperones have been found to mediate different stages of chloroplast import, in similar fashion to chaperone involvement in mitochondrial import. Cytosolic factors such as Hsp90, Hsp70 and 14-3-3 may assist preproteins to reach the TOC complex at the chloroplast surface, preventing their aggregation or degradation. Chaperone involvement in the intermembrane space has also been proposed, but remains uncertain. Preprotein translocation is completed at the trans side of the inner membrane by ATP-driven motor complexes. A stromal Hsp100-type chaperone, Hsp93, cooperates with Tic110 and Tic40 in one such motor complex, while stromal Hsp70 is proposed to act in a second, parallel complex. Upon arrival in the stroma, chaperones (e.g., Hsp70, Cpn60, cpSRP43) also contribute to the folding, assembly or onward intraorganellar guidance of the proteins. In this review, we focus on chaperone involvement during preprotein translocation at the chloroplast envelope. This article is part of a Special Issue entitled: Protein Import and Quality Control in Mitochondria and Plastids.

1. Introduction

Plastids are a diverse group of organelles found ubiquitously in plant cells [1,2]. Chloroplasts, the most prominent members of the plastid family, contain the green pigment chlorophyll and are responsible for the reactions of photosynthesis, as well as sundry important biosynthetic functions. Plastids entered the eukaryotic lineage through endosymbiosis, and have evolved from an ancient photosynthetic prokaryote similar to extant cyanobacteria [3,4]. While plastids retain a functional endogenous genetic system, the plastid genome is greatly reduced and typically encodes just ~100 different proteins [5,6]. Most (>90%) of the ~3000 different proteins that are needed to develop a fully-functional chloroplast are encoded in the nucleus and synthesized on free cytosolic ribosomes [7,8].

Typically, nucleus-encoded chloroplast proteins are synthesized in precursor form, each one having an amino-terminal targeting signal called a transit peptide. These precursors, or preproteins, are transported into the organelle post-translationally, in an energy-consuming process termed chloroplast protein import. Import is mediated by hetero-oligomeric protein complexes in the outer and inner envelope membranes that surround each plastid; these complexes are termed, respectively, TOC and TIC (*T*ranslocon at the *O*uter/*I*nner envelope membrane of *C*hloroplasts) [9–12]. Once a preprotein arrives in the chloroplast interior (the stroma), the transit peptide is proteolytically removed by the stromal processing peptidase (SPP), allowing the protein to assume its functional conformation or engage one of several internal sorting pathways [12–14].

Chloroplast import bears considerable similarity to mitochondrial protein import, which is mediated by translocon complexes termed TOM and TIM (*T*ranslocase of the *O*uter/*I*nner *M*itochondrial membrane) [15–17]. In both cases, preproteins are threaded through the membranes in an unfolded state, amino-terminus first. Both import systems comprise multiple preprotein receptors that project large domains into the cytosol, both possess channel components in the outer and inner membranes, and both are powered, to a greater or less extent, by ATP hydrolysis (see below). However, the principal components of the TOC/TIC and TOM/TIM systems are not closely related. The core components of the TOC complex are Toc159, Toc34 and Toc75 (the numbers indicate size in kD). The first two are receptor components that mediate transit peptide recognition via their cytosolically-oriented GTPase domains, while Toc75 forms a β-barrel channel for preprotein conductance. Electrophysiological analysis of the Toc75 channel indicated a narrow pore ~14 Å in diameter, flanked on either side by

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* Corresponding author. Tel.: +44 116 223 1296; fax: +44 116 252 3330.
E-mail address: rpj3@le.ac.uk (P. Jarvis).

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two wider vestibules [18]. Such a pore would be wide enough only to accept largely unfolded preprotein clients. However, the successful import of a 6.5 kD (23 Å in diameter) tightly-folded, internally-crosslinked protein domain [19] suggests either that the pore is somewhat larger than the aforementioned estimate, or that the channel has a degree of flexibility. Critical components of the TIC apparatus include Tic110 and Tic40, the roles of which will be discussed later.

As already mentioned, protein import into chloroplasts is an energy-dependent process. According to energy requirements determined in vitro, three distinguishable stages of import have been defined. Firstly, the binding of preproteins with the TOC receptors is a reversible and energy-independent step called energy independent binding [20]. Subsequently, initial translocation leads to the formation of an early import intermediate. This irreversible second step requires GTP (for the receptors) and a low concentration of ATP ($\leq$100 μM) in the intermembrane space [21–23]. Finally, preproteins are completely translocated into the stroma at the expense of high concentrations of ATP (~1 mM) in the stroma [24]. The latter energy requirement is attributed to stromal ATPases [25].

The presumed need for preproteins to be in a largely unfolded state during import is dictated by physical characteristics of the import machinery, as discussed earlier, and this in turn necessitates the involvement of molecular chaperones — a diverse group of factors that facilitate folding processes and conformational changes in other proteins [26]. In fact, a variety of different chaperones are required during chloroplast protein import, and these are employed at different stages in the process: in the cytosol following ribosomal release, to prevent misfolding or aggregation of preproteins and to guide them to the chloroplast surface; during the import process itself, to maintain translocation competence of the preproteins and to drive transport at the expense of ATP hydrolysis; and, following the completion of import, to assist with folding, assembly or onward transport to internal destinations. In this review, we will touch on all of these aspects, focusing in particular on chaperone involvement during envelope translocation.

2. Chaperone involvement in the cytosol

Notwithstanding recent evidence that some chloroplast proteins are translated near the border of chloroplasts in the green alga, *Chlamydomonas reinhardtii*, suggesting mRNA transport as a component of the overall targeting scheme [27], chloroplast protein import is generally considered to be a post-translational process (in contrast with signal recognition particle [SRP]-dependent translocation into the endoplasmic reticulum, for example, which is co-translational). Thus, cytosolic factors are required to facilitate the passage of preproteins from the ribosome to the chloroplast surface, and to prevent their aggregation or premature degradation [28–30]. The transit peptide, as the first part of the preprotein to emerge from the ribosome, plays a critical role in the interactions with such components.

Transit peptides are to a large extent responsible for the targeting properties of chloroplast preproteins. Indeed, they are very effective at mediating the import of heterologous passenger proteins into chloroplasts [31,32]. And yet, despite the apparent specificity of the chloroplast import process, transit peptides are remarkably diverse in both length and sequence [33,34]. They vary from 20 to >100 residues, are rich in hydroxylated residues, and are deficient in acidic residues giving them a net positive charge. In this respect, transit peptides are rather similar to the functionally-analogous presequences of mitochondrial preproteins (raising puzzling questions about how organellar targeting specificity is achieved in plants [35,36]). While mitochondrial presequences share a characteristic secondary structure (they form amphipathic helices that are important for interaction with receptors of the TOM machinery [37]), chloroplast transit peptides do not seem to possess this property [38,39]. Instead, it has been hypothesized that they specifically evolved to have "perfect random coil" properties, perhaps to aid interaction with cytosolic factors [40].

Hsp70 (*H*eat-shock *p*rotein, *70* kD) is one of the chaperones thought to facilitate the cytosolic phase of chloroplast protein transport. Most chloroplast transit peptides are predicted to possess at least one Hsp70 binding site, while direct interactions between Hsp70s and transit peptides have been demonstrated [41–44]. However, the importance of such Hsp70 binding for protein import remains uncertain, as it is not essential for protein translocation in vitro [45,46]. Moreover, a recent study showed that cytosolic Hsp70 associates with accumulated precursors that are targeted for degradation via the ubiquitin proteasome system [30], indicating that Hsp70 binding does not necessarily serve to escort preproteins to the chloroplast surface. Nevertheless, Hsp70 does appear to play a role in protein import in cooperation with other cytosolic factors, such as 14-3-3 (see below; Fig. 1). It is conceivable that different isoforms of Hsp70 are responsible for these different functions.

The 14-3-3 protein family includes regulatory molecules and chaperones that specifically bind to phosphorylated proteins in order to mediate various signal transduction processes, as well as protein translocation [47]. Many chloroplast transit peptides contain a 14-3-3-binding phosphopeptide motif [28,48]. It was reported that 14-3-3 can form a "guidance complex" together with Hsp70 and preproteins, and that this significantly increases in vitro import efficiency for certain, phosphorylatable preproteins [28]. The 14-3-3-containing guidance complex was also hypothesized to play a role in determining the specificity of targeting to chloroplasts versus mitochondria in plants, as 14-3-3 cannot bind plant mitochondrial preproteins [28]. However, mutation of the putative 14-3-3-binding site in transit peptides did not affect import efficiency or fidelity in vivo [49,50], indicating that the 14-3-3 guidance complex system is dispensable. It is possible that this mechanism is important only under certain conditions; it was recently reported that the loss of a kinase thought to be responsible for transit peptide phosphorylation results in an inefficient de-etiolation response [51].

Differentiating between two distinct, endosymbiotically-derived organelles (i.e., chloroplasts and mitochondria) is a unique problem faced by protein transport systems in plant cells. Related to this issue, perhaps, is the fact that the protein import receptors in plant mitochondria are significantly different from those in yeast or animal mitochondria, as well as from those in chloroplasts [17,36]. In spite of these receptor differences, some chloroplast preproteins can be efficiently imported into plant mitochondria in vitro, but not in vivo [52]. This implies that special mechanisms are employed to achieve import specificity in vivo, and that components of such mechanisms are absent or inactive in vitro. Aside from the 14-3-3 guidance hypothesis discussed above, one strategy that might contribute to targeting specificity is mRNA transport towards the target destination, such that preproteins are produced only at the periphery of the correct organelle [27,53,54]. However, the general significance of mRNA targeting in plants remains to be seen.

In mitochondrial protein import in animal cells, Hsp90 is an additional chaperone involved in cytosolic guidance, directing some preproteins to the Tom70 receptor [55]. Similarly, Hsp90 has also been implicated in the delivery of certain preproteins to chloroplasts as part of a second guidance complex, which was recently reported to also involve the cochaperone Hop (*H*sp70/Hsp90-*o*rganizing *p*rotein) and the immunophilin FKBP73 [56–58]. There are two important differences between this guidance complex and the one discussed earlier: firstly, Hsp90 binds to preproteins that are not necessarily phosphorylated; secondly, unlike the 14-3-3 complex which carries preproteins directly to the Toc34 receptor, Hsp90 employs Toc64 (see below) as an initial docking site before preproteins are passed on to Toc34 [56] (Fig. 1). However, preproteins proposed to follow the Hsp90–Toc64 pathway were found to be imported with normal efficiency into chloroplasts that lack Toc64 protein [59,60], indicating that this putative targeting mechanism is also not essential. It is conceivable that such