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Toward a unified model of the action of CLP/HSP100 chaperones in chloroplasts

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In chloroplasts, Hsp70 and Hsp100 chaperones have been long suspected to be the motors that provide the necessary energy for the import of precursor proteins destined to the organelle. The chaperones associate with the import translocon and meet the transit peptides as they emerge through the channel. After decades of active research, recent findings demonstrated that Hsp100 chaperones recognize transit peptides both in vitro and in vivo. Moreover, Hsp70 also plays a part in precursor import. The updated model of protein translocation into chloroplasts now presents new questions about the role of the chaperones in the process.

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The term molecular chaperone was first coined to describe a group of proteins that assist in the correct folding of other polypeptide chains.[1] Eventually, other functions were discovered such as (dis) assembly of protein complexes, translocation across membranes and degradation of proteins damaged beyond repair, to name a few. All in all, molecular chaperones regulate protein levels within a normal range. For this reason, they are recognized as being key players in maintaining cellular proteostasis.[2]

In chloroplasts, members of the Hsp60 (chaperonins), 70 (DnaK-like), 90, 100 (Clp) as well as other small Hsps were identified.[3] In particular, the stromal Clp/Hsp100 family has received much attention due to its central role in protein degradation and precursor import.

ClpA, the first member of Clp/Hsp100 proteins, was discovered in *Escherichia coli* as part of a bipartite proteolytic complex.[4] One component is made by ClpP, which assembles into two heptameric rings that enclose a chamber with proteolytic activity. Polypeptides subjected to degradation cannot enter the chamber in a folded state. So the ClpA chaperone selects and unfolds the target in an ATP-dependent manner and presents it to the ClpP protease. The Clp complex has also been found in plants. In chloroplasts of *Arabidopsis thaliana*, the Clp/Hsp100 group is represented by ClpB3, ClpC1, ClpC2 and ClpD while the protease group includes five different ClpP proteases (ClpP1, 3–6) and four ClpP-like proteins with no protease activity (ClpR1–4).[3]

To Degrade or to Import?

The Clp complex is one of the three families that play a crucial role in protein degradation in chloroplasts (the others being the FtsH and Deg/HtrA families). The Clp protease has an architecture that resembles that of its bacterial counterpart: a barrel-shaped ClpP/R protease core capped by Clp/Hsp100 chaperones.[5,6] As is the case for *E. coli* ClpA, recombinant ClpC2 and ClpD self-assemble into hexamers and possess ATPase and chaperone activity.[7] The protein expression profile of Clp/Hsp100 chaperones is constitutive, which early prompted the notion that they perform maintenance duties.[8] In *A. thaliana*, while insertional mutants in the *clpc2* or *clpd* genes have no obvious phenotype, knockout plants in *clpc1* are pale green, have retarded growth and their chloroplasts possess ultrastructural defects.[9-11]

All this evidence established chloroplastic Clp/Hsp100 proteins as stromal housekeeping chaperones.

In addition, Moore and Keegstra discovered that the Clp/Hsp100 homolog from pea (Hsp93) could be detected at the inner membrane by polyclonal antibodies raised against envelope proteins.[12] Hsp93 was associated with the import machinery, while Hsp70 was not (although this finding was later rebutted, see below).[13,14] This led to the conclusion that Hsp93 was providing, at least in part, the driving force for translocation. Other lines of research supported the hypothesis. Our laboratory showed that precursors bearing a mutated transit peptide with diminished affinity for Hsp70 were still efficiently imported.[15] Chloroplasts from *A. thaliana* knockout lines in the *clpc1* gene showed import defects.[10,16] Recently, we showed that ClpC1/2 and ClpD from *A. thaliana* could be found at the inner membrane and stably interacted with transit peptides in vitro and in vivo.[7]

It can be safely stated that chloroplastic Clp/Hsp100 chaperones are involved in protein quality control and import. But a unified model for their mode of action raises important questions. For example, when a Clp/Hsp100 chaperone interacts with a transit peptide engaged in translocation, how is it regulated that the ClpP core does not bind to the chaperone moiety and degrades the precursor? How do Clp/Hsp100 chaperones discriminate against polypeptides destined for import or degradation? We propose several hypotheses that could shed some light into this gray area:

Regulatory protein interaction. *(1) Modulator proteins.* A few other proteins interact with the Clp complex. *E. coli* ClpS is a modulator protein that is involved in substrate recognition. It recognizes targets that follow the N-end rule and presents them to the ClpA chaperone.[17] ClpS is also found in chloroplasts but its function is still unknown. It may be responsible for selecting the appropriate substrates for the ClpP/R complex. Whether ClpS selectively binds to precursors or damaged proteins is not known. In *A. thaliana*, two other proteins named ClpT1 and ClpT2, affect the formation of the Clp complex. By homology modeling, it was found that ClpT proteins may bind to the ClpP subunits, thus preventing Clp/Hsp100 chaperones to interact with the proteolytic core.[6]

(2) Interaction with the translocon. Hsp93 interacts with Tic40 and stimulates its ATPase activity, which indicates that Tic40 causes some type of structural change in the chaperone.[18] It could be possible that the association of the chaperones with translocons components inhibits binding to ClpP and, consequently, precursor degradation.

Sequence tags. Clp/Hsp100 chaperones may discriminate between precursors and proteins destined for degradation by recognizing different sequence determinants in the target protein. This would imply that distinctive sequence characteristics influence the association to the ClpP core. To explore this possibility, it is necessary to determine if Clp/Hsp100 binding sites in precursors or in terminally-damaged proteins are different. However, no conclusive information exists about Clp/Hsp100 substrates, let alone about specific tags for protein degradation. On the other hand, sequence determinants in transit peptides for Clp/Hsp100 binding have not been determined.

Distinct populations of chaperones. It has been assumed that the populations of chaperones at the inner membrane and at the stroma are equal. However, it cannot be ruled out that Clp/Hsp100 chaperones located at the inner membrane are structurally different than their stromal counterparts. Post-translational events may direct their final destination, substrate affinity and protease interaction. Interestingly, a C-terminal truncated variant of ClpD interacted with a model transit peptide with greater affinity than the full-length protein.[7]

Many are Better than One

Initial efforts to elucidate the role of Hsp100 proteins in precursor import in *A. thaliana* were directed to ClpC1. This is not surprising as *clpc1* knockout plants present a sick phenotype and import defects while *clpc2* or *clpd* knockout plants do not. But we have recently shown that ClpC2 and ClpD can interact with precursor proteins.[7] It can be argued that all three chaperones can fulfill the same role. Not only single mutants in the corresponding genes are viable but also, their expression patterns are very similar. This would rule out tissue- or developmental-specific roles. Also, overexpressing ClpC2 in a Δ*clpc1* background restores the wild type phenotype.[16] The functional significance of the multiple homologs of Clp/Hsp100 chaperones is not clear. An exciting hypothesis to explore would be to determine if they can recognize different subsets of substrates, in normal or stressful conditions.

If redundancy among members of the same family is puzzling, then going up one level adds an extra layer of complexity. It was recently found that Hsp70 chaperones have overlapped functions with Clp/Hsp100 proteins. Work in moss and *A. thaliana* showed that Hsp70 chaperones function as part of the chloroplast protein import machinery.[19,20] An *A. thaliana* double mutant in the dominant paralogs of Hsp70 and Hsp100 (CPHSC70-1 and HSP93-V, respectively) have a rate of import more diminished than that observed for the single mutants, indicating that the chaperones might work separately. The current model of protein import into chloroplasts now places both systems working in parallel. But since *clpc1 clpc2* and *hsp70-1 hsp70-2* double mutants are not viable, it is hard to know if one system can take the entire load if the other is not available.

Why so many chaperones are found to be involved in precursor translocation in chloroplasts? One explanation would be that, given that chaperones are known for their substrate promiscuity, both Hsp100 and Hsp70 proteins have the ability to intercept any precursor entering the organelle. That being the case, then transit peptide recognition could lay mostly on the components of the translocon of the outer membrane.

Concluding Remarks

In recent years, there have been great advances in the fields of protein degradation and precursor import in chloroplasts. They are intimately intertwined by the central role molecular chaperones play in both processes. Even though both areas

have still many unanswered questions on their own, researchers must also concentrate in the link joining them together. Novel insights that explain how chaperones regulate the fate of precursors or terminally damaged proteins or how the different chaperone systems work in unison will definitely lead to a better understanding of chloroplast biogenesis and maintenance.

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