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Requirements for a conservative protein translocation pathway in chloroplasts

Lea Vojta, Jürgen Soll*, Bettina Bölter

University of Munich, Botany, Menzinger Str. 67, 80638 Munich, Germany

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Abstract The chloroplast inner envelope translocon subunit Tic110 is imported via a soluble stromal translocation intermediate. In this study an in-organellar import system is established which allows for an accumulation of this intermediate in order to analyze its requirements for reexport. All results demonstrate that the re-export of Tic110 from the soluble intermediate stage into the inner envelope requires ATP hydrolysis, which cannot be replaced by other NTPs. Furthermore, the molecular chaperone Hsp93 seems prominently involved in the reexport pathway of Tic110, because other stromal intermediates like that of the oxygen evolving complex subunit OE33 (iOE33) en route to the thylakoid lumen interacts preferentially with Hsp70.

Keywords: Chloroplast; Protein import; Tic110; Hsp93; Molecular chaperones

1. Introduction

Chloroplasts must import most of their protein constituents in a posttranslational event from the cytoplasm [1]. At the same time, a complex sorting machinery must guarantee that chloroplast destined preproteins end up in the correct organellar subcompartment. The chloroplast is subdivided into three distinct membrane systems: the outer and the inner envelope membranes as well as the internal thylakoid network which houses the photosystems. In addition, three soluble compartments can be distinguished: the space between the two envelope membranes, the stroma containing all the enzymes involved in primary carbon metabolism, and the thylakoid lumen.

The standard import pathway for preproteins has been described in some detail [2]. In general, the chloroplast targeted protein, e.g. the precursor of the stroma localized small subunit of the RuBisCO (pSSU) or the thylakoid localized chlorophyll *a/b* binding protein LHC (pLHC) contain an N-terminal targeting signal which is proteolytically cleaved during or shortly after translocation [3]. As soon as the preproteins have completely reached the stromal space they are folded and assembled with the help of molecular chaperones or further routed to the thylakoids. In the case of LHC this requires evolutionary conserved subunits of the Sec pathway, namely the Cp54, an SRP54 homolog, an SRP receptor homolog, SecY and a plant specific factor, Cp43 [4].

Much less is known how proteins are targeted to the inner envelope of chloroplasts. In general, inner envelope localized preproteins are also synthesized with an N-terminal cleavable presequence, though exceptions have been described for Tic32 [5] and QORH [6], which are targeted by internal sequence information. The targeting of those inner envelope proteins which contain a cleavable presequence and seem to follow the general import pathway falls into two categories. One route comprises preproteins which seem to be inserted by a stop transfer pathway, i.e. they are recognized during translocation across the inner envelope membrane and released by an unknown mechanism directly into the inner envelope, a pathway which is well described for mitochondria and which uses the Tim23 translocon [7]. The other route is named the conservative sorting pathway. Here, preproteins are first routed into the matrix (stroma) space, processed, and then become re-exported into the inner membrane [8].

Due to the endosymbiotic origin of mitochondria and chloroplasts [9] this pathway superficially resembles a posttranslational translocation pathway into the plasma membrane of bacteria and was named accordingly [10]. In chloroplasts very little is known about the molecular characteristics of those pathways, though both seem to exist. While some inner envelope preproteins seem to import without a soluble stromal intermediate, like the phosphate–triose–phosphate-translocator [11], others have been shown to accumulate in the stroma, like Tic110 or Tic40, before being inserted into the inner envelope [12,13].

Here we used pTic110 and a chimeric preprotein consisting of the N-terminal membrane anchor region of Tic110 fused to the mature portion of SSU to study the accumulation and re-export requirements in an isolated organellar system. We show that re-export requires the presence of ATP or an ATP-regenerating system inside chloroplasts that cannot be replaced by other NTPs. Furthermore, stromal intermediates are associated with specific molecular chaperones which most likely catalyze their insertion process into the inner envelope.

2. Material and methods

2.1. In vitro transcription and translation

The coding regions for Tic110 from *Pisum sativum* and pSSU-110N-SSU were cloned into the vector pET21d under the control of the T7 promoter. The coding region for the hybrid construct pSSU-Tic110N-SSU [13] contains the presequence of pSSU (aa 1–64), the N-terminal

*Corresponding author. Fax: +49 89 17861185.
E-mail addresses: soll@lmu.de (J. Soll), boelter@lrz.uni-muenchen.de (B. Bölter).

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part of Tic110 (aa 37–272 bp of the original clone) and the mature part of SSU (aa 58–188). Tic110 sequence originates from *P. sativum* and pSSU from *Nicotiana sylvestris* (accession number X53426). For the purpose of enhancing the radioactive signal after in vitro translation, additional six methionines were cloned at the C-terminal end of each of the proteins. Transcription of linearized plasmids was carried out in the presence T7 polymerase using the chemicals from MBI Fermentas. Translation was carried out using the Flexi Rabbit Reticulocyte Lysate System (Promega) in the presence of ^{35}S-methionine/cysteine mixture (MGD1) for radioactive labeling. After translation, the reaction mixture was centrifuged at 50 000 × *g* for 20 min at 4 °C and the post-ribosomal supernatant was used for import experiments.

2.2. Chloroplast isolation and protein import

Chloroplasts were isolated from leaves of 9–11 days old pea seedlings (*P. sativum* var. Arvica) and purified through Percoll density gradients as described [14]. Prior to import, ATP was depleted from chloroplasts and the translation product. A standard import reaction contained chloroplasts equivalent to 15–20 μg chlorophyll in 100 μl import buffer (330 mM sorbit, 50 mM HEPES/KOH pH 7.6, 3 mM $MgSO_4$, 10 mM Met, 10 mM Cys, 20 mM K-gluconate, 10 mM $NaHCO_3$, 2% BSA (w/v)), up to 3 mM ATP and maximal 10% (v/v) ^{35}S-labelled translation products. Import reactions were initiated by the addition of translation product and carried out for 20 min at 25 °C, unless indicated otherwise. Reactions were terminated by separation of chloroplasts from the reaction mixture by centrifugation through 40% (v/v) Percoll cushion. Chloroplasts were washed once, lysed in 10 mM HEPES/KOH pH 7.6 for 30 min on ice and separated into membrane and soluble fractions by centrifugation at 265 000 × *g* for 10 min at 4 °C. Import products were separated by SDS–PAGE and radiolabelled proteins analyzed by a phosphor-imager or by the exposure on X-ray films.

For the purpose of investigating the energy requirement of Tic110 and pS-110N-SSU on their reexport import pathway radioactively labeled precursors were incubated with 20 μg chloroplasts in the import mix containing 3 mM ATP for 2 min at 25 °C. Afterwards chloroplasts were pelleted and resuspended in the new import mix containing either ATP, GTP or PEP at the final concentration of 3 mM, or the non-hydrolysable ATP analog AMP-PNP at the final concentration of 5 mM. It was incubated in fresh import mix on ice for 5 min and subsequently 10 min at 25 °C. All incubations were performed in the dark to diminish the influence of internally produced ATP. Afterwards, chloroplasts were pelleted 1 min at 1500 × *g*, washed, and separated into soluble and membrane fractions.

2.3. Coimmunoprecipitation of Tic110 and pSSU-Tic110N-mSSU soluble stromal intermediates

After 3 min of Tic110 and pSSU-Tic110N-mSSU import and 10 min of pOE33 and pSSU control import, chloroplasts were reisolated on a Percoll cushion, washed and separated into the membrane and soluble fractions. The soluble fraction was diluted 1:1 in 2×IP buffer (25 mM HEPES/KOH pH 7.6 and 150 mM NaCl) with addition of 0.3% decylmaltoside (DM) and 0.5% egg albumin and incubated with 5 μl primary antiserum α-Hsp70, α-Hsp93 or α-OEP16, rotating for 90 min at RT, followed by subsequent incubation with 20 μl of Protein A-Sepharose for another 90 min at RT. The matrix was subsequently washed and elution was performed using Laemmli sample buffer. Flow through, washes and elution fractions were analyzed by SDS–PAGE and exposed on X-ray film.

3. Results and discussion

The aim of this study was to establish an in-organellar system, which allows identifying requirements for the re-export of stromal localized translocation intermediates to the inner envelope of chloroplasts. As a model substrate we used the integral translocon subunit Tic110, which had been shown before to form such a stromal intermediate [13]. Tic110 is anchored to the inner envelope by two hydrophobic α-helices located at the N-terminus of the protein. These are required for proper localization [15]. In order to determine an effect of the transit peptide on Tic110 translocation we exchanged it with the transit peptide of SSU. In addition, the C-terminus of Tic110, which structure is not well-defined, was exchanged by the mature part of SSU. Earlier experiments had indicated that this chimeric protein pS-Tic110N-SSU is imported into chloroplasts and targeted to the inner envelope membrane [13].

In order to establish the in-organellar system we studied the time dependence of the import reaction for both pTic110 and pS-Tic110N-SSU (Fig. 1). After import, chloroplasts were lysed in hypotonic buffer and proteins were separated into a soluble and a membrane fraction. Already at the shortest time point tested (1 min) we could detect the imported mature form of the two preproteins in both the soluble and the membrane fraction. The soluble forms of Tic110 and Tic110N-SSU reached a maximum between 2.5 and 5 min, after which they decreased again, indicating that they become integrated into the membrane (Fig. 1, graph). We also continuously observed a higher ratio of membrane bound to soluble Tic110N-SSU than for the wild type Tic110, indicating that the kinetics are shifted towards efficient import into the stroma while the re-export rate may be decreased in this chimeric preprotein.

Next, we investigated if the soluble preprotein is on a productive translocation pathway, i.e. if completion of translocation was energy dependent. First, chloroplasts were incubated with precursor proteins for 2 min at 25 °C in the presence of 3 mM ATP. Then, chloroplasts were separated from the import mixture by centrifugation and subsequently resuspended in fresh import buffer containing different additions, e.g. ATP, GTP, phosphoenolpyruvate or the non-hydrolysable ATP analogue AMP-PNP (Fig. 2). The re-export of Tic110

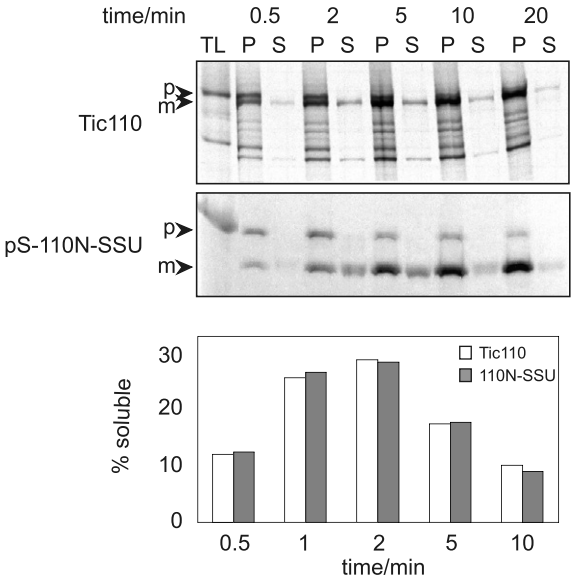

Fig. 1. Time-scale of Tic110 and pS-110N-SSU import. [^{35}S]-pTic110 and [^{35}S]-pS-110N-SSU were incubated with intact pea chloroplasts under standard conditions, for 0.5–20 min at 25 °C. After import chloroplasts were separated into the pellet (P) and soluble (S) fractions. Lane 1 indicates 1/10 of the translation product (TL) used for the import reaction. Precursor (p) and mature (m) forms of Tic110 and pS-110N-SSU are indicated by arrows. Bottom panel: quantification of the soluble intermediates.

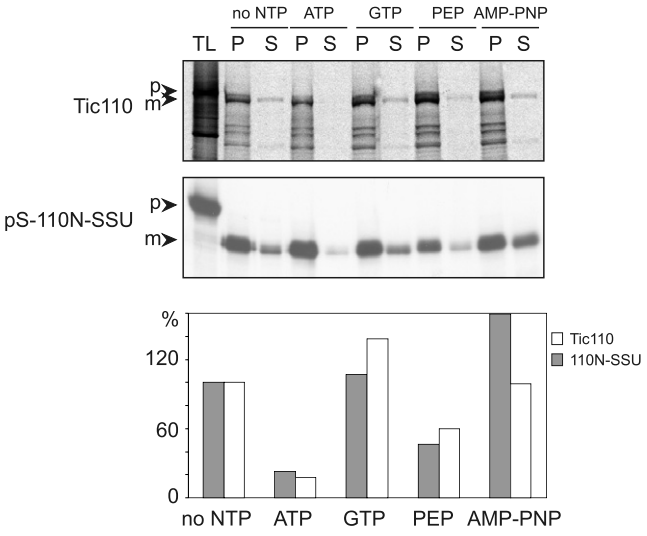

Fig. 2. Energy requirement for the re-export of Tic110 and S-110N-SSU. Both radioactively labeled proteins were imported in separate standard import reactions at 25 °C in the presence of 3 mM ATP. The import was arrested after 2 min and the chloroplasts were transferred into a new import mix containing no NTPs (lanes 2 + 3), 3 mM ATP (lanes 4 + 5), 3 mM GTP (lanes 6 + 7), 3 mM PEP (lanes 8 + 9) or 5 mM AMP-PNP (lanes 10 + 11). Samples were incubated for 5 min on ice followed by 10 min of import at 25 °C. Chloroplasts were subsequently separated into the pellet (P) and soluble (S) fractions. Lane 1 represents 1/10 of the translation product (TL) used for the import reaction. Bottom panel: quantification of the soluble intermediates.

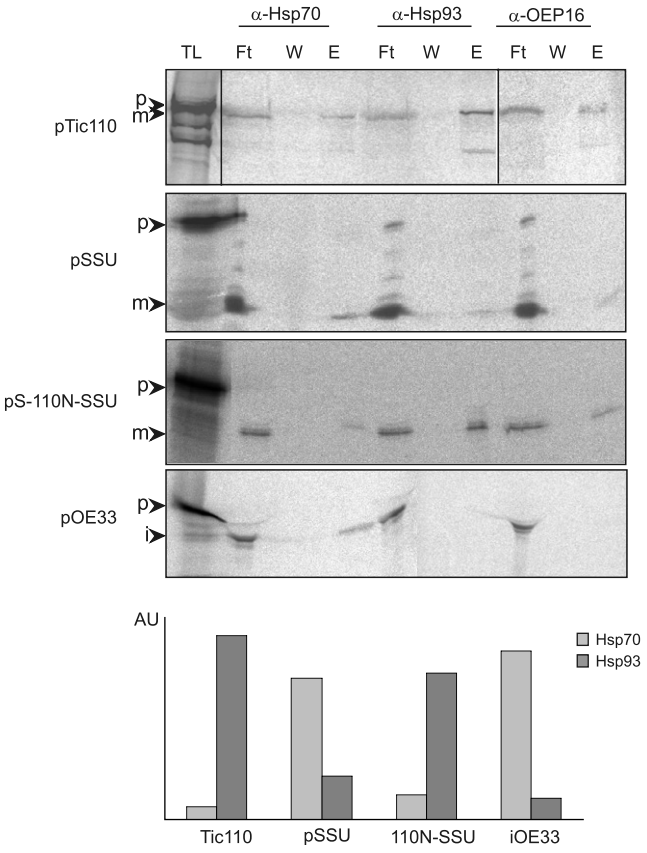

Fig. 3. Co-immunoprecipitation of stromal intermediates of Tic110, 110N-SSU, Tic40 and OE33 by stromal chaperones. [^{35}S]-pTic110, [^{35}S]-pSSU, [^{35}S]-pS-110N-SSU and [^{35}S]-pOE33 were incubated with intact pea chloroplasts corresponding to 20 μg chlorophyll for 3.5 min at 25 °C in the presence of 3 mM ATP. After import, chloroplasts were reisolated on a Percoll cushion, washed and separated into the membrane and soluble fractions. The soluble stromal intermediates were dissolved in IP buffer and incubated with antibodies raised against stromal chaperones: α-Hsp70, α-Hsp93 and α-OEP16 as a control, 0.5% egg albumin, and 0.3% decylmaltoside for 90 min at RT. Antibodies were bound to Protein A-Sepharose for 1 h at RT. The flow through after the incubation with Protein A-Sepharose (FT), the third wash (W) and the elution with Laemmli sample buffer (E) were analyzed by SDS–PAGE. TL indicates 1/10 of the translation product used for each experiment. Bottom panel: quantification of the soluble intermediates in the eluate fractions. Values have been normalized to the OEP16 immunoprecipitation representing the unspecific background.

or Tic110N-SSU was dependent on ATP, as judged from their disappearance from the soluble fraction (Fig. 2, see quantification in lower panel). In the absence of ATP or in the presence of the non-hydrolysable analogue AMP-PNP, most of the import intermediate remained in the soluble fraction. The energy requirement for the re-export reaction seems specific for ATP since GTP could not substitute for ATP, while PEP, which is converted by pyruvate kinase in chloroplasts to ATP and pyruvate, clearly supported the re-export reaction, though to a lesser extent than ATP itself. This is most likely due to the fact that by this indirect ATP supply only limiting amounts of the nucleotide are produced which slows the re-export action down.

The requirement for ATP hydrolysis to catalyze the re-export reaction clearly suggested that further components could be involved in the pathway, e.g. molecular chaperones. To study this, translocation experiments of preproteins into chloroplasts were carried out for 3.5 min at 25 °C in the presence of ATP. Chloroplasts were then recovered from the import reaction, lysed with hypotonic buffer to retain an optimal chaperone–preprotein-interaction and the soluble fraction was subjected to co-immunoprecipitation using antisera against Hsp93, Hsp70 and OEP16 as a negative control, respectively (Fig. 3). We tested different precursor proteins, i.e. pSSU which is localized in the stroma, pOE33, which is localized to the thylakoid lumen and forms a soluble translocation intermediate in the stroma, and the two Tic110 preproteins. Processed mature SSU, and the import intermediate iOE33 interacted primarily with Hsp70, while only very little of either protein could be immunoprecipitated by Hsp93 antiserum. In contrast, Tic110 and Tic110N-SSU interacted primarily with Hsp93 while very little preprotein could be co-immunoprecipitated by Hsp70 antisera. The C-terminus of Tic110 in the inner envelope has been reported to interact with Hsp93. Because the hybrid protein Tic110N-SSU does not contain this peptide region anymore, we conclude that the soluble Hsp93-Tic110 hetero-oligomeric complex is a *bona fide* intermediate in the import pathway of Tic110. This notion is supported by similar results obtained for Tic40 (data not shown). The small portion of Tic110 and Tic110-SSU soluble intermediates observed on Hsp70 are most probably due to the tendency of Hsp70 to bind more or less unspecifically to all unfolded polypeptides. A recent publication by Kovacheva et al. describes a transgenic Arabidopsis line in which both alleles for Hsp93 are interrupted [16]. The amount of Hsp93 protein in this knockdown mutant is reduced to about 20%. These plants exhibit a chlorotic phenotype but show little influence in the protein presence of Tic110 and Tic40, indicating that the residual

Hsp93 is sufficient to support export of Tic110 or that other chaperones can assist the function of Hsp93 in this conservative sorting pathway. On the other hand, an increased expression rate of Tic110 in these mutants might also cause a seemingly unchanged amount of protein, but the expression level is not documented [16].

4. Conclusion

The conservative sorting pathway of the inner envelope localized chloroplast translocon subunit Tic110 involves the stromal chaperone Hsp93 and requires ATP, most likely for the action of the molecular chaperone. However, we cannot exclude that further steps in the re-export pathway need ATP. Our results clearly promote our understanding on the molecular basis of the re-export pathway, which was previously thought to be independent on any proteinaceous factors and to occur spontaneously into the inner envelope. In these studies [12] isolated inner envelope vesicles were incubated with in vitro translated Tic110 in a wheat germ lysate. Association of Tic110 with the inner envelope independent of an exogenous energy source was observed, which was also resistant to the extraction at pH11. The two different systems described here and in the manuscript by Li and Schnell [12] are difficult to compare, but the latter study used an extremely different ratio of preproteins to inner envelope than in our in-organellar system, therefore it could be out of the biochemically defined linear range for the reaction partner inner envelope resulting in non-selective adhesion or insertion. On the other side, resistance to extraction with Na_2CO_3 does not necessarily mean productive transmembrane insertion, but could also be caused by strong hydrophobic interaction of the transmembrane helices with the surface of the lipid bilayer. The positive evidence presented here for the molecular requirements of the re-export pathway of Tic110 strongly argues against the spontaneous uncatalysed process. Spontaneous insertion had been described in bacteria for the MscL protein, which is now known to require YidC [17].

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