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Review

Protein import into chloroplasts: The Tic complex and its regulation

Erika Kovács-Bogdán [a,b], Jürgen Soll [a,b], Bettina Bölter [a,b,*]

[a] Department Biologie I-Botanik, Ludwig-Maximilians-Universität, Großhadernerstr. 2-4, D-82152 Planegg-Martinsried, Germany
[b] Munich Center for Integrated Protein Science CiPSM, Ludwig-Maximilians-Universität, Feodor-Lynen-Strasse 25, D-81377 Munich, Germany

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ABSTRACT

Chloroplasts like mitochondria were derived from an endosymbiontic event. Due to the massive gene transfer to the nucleus during endosymbiosis, only a limited number of chloroplastic proteins are still encoded for in the plastid genome. Most of the nuclear-encoded plastidic proteins are post-translationally translocated back to the chloroplast via the general import pathway through distinct outer and inner envelope membrane protein complexes, the Toc and Tic translocons (*T*ranslocon at the *o*uter/*i*nner envelope membrane of *c*hloroplasts). Eight Tic subunits have been described so far, including two potential channel proteins (Tic110 and Tic20), the "motor complex" (Tic40 associated with the stromal chaperone Hsp93) and the "redox regulon" (Tic62, Tic55, and Tic32) involved in regulation of protein import via the metabolic redox status of the chloroplast. Regulation can additionally occur via thioredoxins (Tic110 and Tic55) or via the calcium/calmodulin network (Tic110 and Tic32). In this review we present the current knowledge about the Tic complex focusing on its regulation and addressing some still open questions.

1. Introduction

Chloroplasts and mitochondria were derived from two independent single endosymbiotic events, respectively. Mitochondria originated from an α-proteobacterium, which was incorporated into an unknown host cell approximately 2 billion years ago. Later, a heterotrophic cell already containing mitochondria took up a cyanobacteria-related organism, the ancestor of chloroplasts, about 1.6–1.5 billion years ago, giving rise to a eukaryotic cell being capable of an autotrophic life style [1,2]. All plastid types most likely evolved from this single endosymbiontic event: the plastids of glaucophytes, red algae, green plants and of their relatives [3–5]. During the endosymbiotic event a massive gene transfer occurred, when most mitochondrial and plastidic genes were transferred to the host cell nucleus. This process can be mimicked experimentally, demonstrating that organellar genes can be transferred to the nucleus [6,7]. As a result, plastids contain about 3000 proteins, but just 50–200 of them are still encoded for in the plastid genome [8,9]. Therefore, the organelles had to develop a system to re-import the necessary proteins from the cytosol back into the organelle and sort them to the correct sub-compartments.

The nuclear-encoded proteins are translated on cytosolic ribosomes, transferred to the organelles and imported across the organelle membranes. These processes share several similarities between mitochondria and plastids: (i) the preproteins engage general chaperones (preventing premature folding of the preprotein), (ii) targeting to both organelles mostly requires an organelle-specific signal (presequence or transit peptide, respectively), and (iii) both organelles developed translocon complexes in the outer and inner membranes, which contains core elements inherited from the bacterial ancestors (for review see [10–12]). The different stages of protein transport processes offer several opportunities for regulation of the system depending on the requirements of the organelles.

Most of the chloroplastic preproteins use the so-called "general import pathway" for their passage into the chloroplast (Fig. 1). They bear an N-terminal transit peptide, which is responsible for the specificity of targeting [13,14]. After translation in the cytosol, preproteins are kept in an import-competent state with the help of chaperone-assisted complexes. So far two different complexes were identified: the so-called "guidance complex" consists of preprotein associated with Hsp70 chaperones, 14-3-3 proteins, and possibly other unidentified components [15]. Some transit peptides contain a specific binding site for the 14-3-3 dimers, being enriched in phophorylatable Ser and Thr residues. These preproteins can associate stronger with 14-3-3 proteins when they are phosphorylated. A small kinase family (in *Arabidopsis thaliana*) was shown to be responsible for this phosphorylation [16,17]. Another cytosolic complex contains Hsp90 and Hsp70 chaperones associated with the preprotein [18].

On the chloroplast surface preproteins are recognized by specific receptors of the Toc complex, which catalyses the translocation across

Abbreviations: CaM, calmodulin; Clp, caseinolytic protease; DEPC, diethylpyrocarbonate; FNR, ferredoxin-NADP$^+$-oxidoreductase; Hip, Hsp70 interacting protein; Hop, Hsp70 and Hsp90 organizing protein; PAO, pheophorbide a oxygenase; Tic, translocon at the inner envelope membrane of chloroplasts; Toc, translocon at the outer envelope membrane of chloroplasts; TPR, tetratricopeptide repeat; Trx, thioredoxin

* Corresponding author. Munich Center for Integrated Protein Science CiPSM, Ludwig-Maximilians-Universität, Feodor-Lynen-Strasse 25, D-81377 Munich, Germany. Fax: +49 89 218074752.
E-mail address: boelter@lrz.uni-muenchen.de (B. Bölter).

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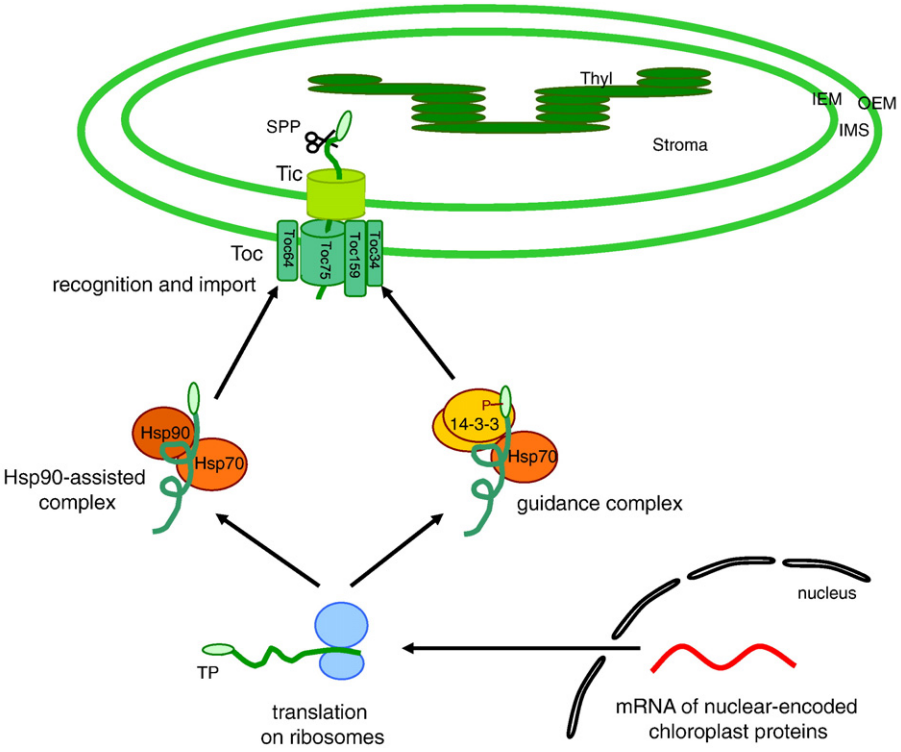

Fig. 1. Schematic illustration of the "general import pathway". Nuclear-encoded chloroplastic proteins are first translated on cytoplasmic ribosomes and then transported to the chloroplast with the help of chaperone-assisted complexes. Toc34 and Toc64 serve as docking sites for the "guidance complex" and the Hsp90-assisted complex, respectively. Following the import across the outer and inner envelope membranes (OEM, IEM) of chloroplasts via the Toc and Tic complexes, the transit peptide (TP) is cleaved off by the stromal processing peptidase (SPP) after successful import into the stroma. IMS: intermembrane space, Thyl: thylakoids.

the outer envelope membrane (Fig. 1). Three components are supposed to catalyse initial recognition: Toc34 serves as a primary receptor for the preprotein, regulated by GTP-binding and phosphorylation [19–21]. The affinity to preproteins is stronger in the GTP-bound form, whereas phosphorylation of Toc34 inhibits GTP-binding and thereby the interaction with the preprotein. Furthermore, it has a higher affinity to the phosphorylated preprotein (associated with the guidance complex). Toc159 was described not just as a receptor in the outer envelope membrane but also as a GTP-driven motor that threads the preproteins into the Toc-channel [22]. In addition, phosphorylation of Toc34 and Toc159 can mediate the dissociation of the Toc complex [23]. Toc64 is a docking site for Hsp90 chaperone-complexed preproteins, which contains three tetratricopeptide repeat (TPR) domains mediating the binding to Hsp90 [18,24,25]. TPR domains are defined by the presence of two antiparallel-helices with a total length of 34 amino acids, usually present in several copies and they are known to mediate protein–protein interactions [26]. The import channel of the Toc complex is formed by a β-barrel protein, Toc75 [27]. It belongs to the Omp85 family of bacterial outer membrane proteins, suggesting that the chloroplast outer envelope originated from the cyanobacterial outer membrane [28–30]. The fifth Toc subunit, Toc12 associates in the intermembrane space with Toc64, Tic22, and an Hsp70 chaperone, thereby creating the so-called intermembrane space complex that is thought to facilitate the efficient and direct translocation of preproteins between the two translocon complexes [31]. For more details about the function of Toc components see Jarvis [11].

The Tic complex is responsible for preprotein translocation across the inner envelope. Eight components have been identified so far: Tic110, Tic62, Tic55, Tic40, Tic32, Tic22, Tic21, and Tic20 (Fig. 2). Tic22 is the only soluble Tic subunit in the intermembrane space, being part of the intermembrane space complex (see above). Two proteins, Tic110 and Tic20 are supposed to form protein translocation channel (s). Tic40, together with the stromal chaperone Hsp93, is believed to provide the driving force (dependent on ATP hydrolysis) for the import as a "motor complex". After the preprotein reached the stroma through the channel with the help of the motor complex, the transit peptide is cleaved off by the stromal processing peptidase. Tic62, Tic55, and Tic32 enable redox regulation of the import via their redox-sensitive groups. This review summarizes our current knowledge of the Tic translocon components and the regulation of protein import at the stage of the inner envelope membrane.

2. Channel-forming components

Tic110 is the most abundant component of the Tic translocon [32–34]. As demonstrated by electrophysiological measurements, the mostly alpha-helical (53%) protein forms a cation-selective channel in isolated inner envelope vesicles and proteoliposomes with an inner pore diameter of 1.7 nm [35,36].

Tic110 is imported into the chloroplast via the general import pathway. Following import into the stroma and processing to its mature size, the soluble intermediate is inserted into the membrane which requires its N-terminal region [34,37]. This re-export into the inner envelope is catalysed by ATP and involves Hsp93 [38].

The extreme N-terminus of Tic110 contains two hydrophobic transmembrane alpha helices [39]. The localization of the hydrophilic rest of the protein was debated: according to one hypothesis this part is soluble, localized in the stroma, and is responsible for chaperone recruitment [40,41]. Lübeck et al. [34] treated intact chloroplasts proteolytically with trypsin (digesting inner envelope proteins only at the intermembrane space side) and described the digestion of this portion of Tic110, indicating that it is located in the intermembrane space. Further analysis revealed that Tic110 even without its N-terminus is able to insert into liposomes and to form a cation-selective channel similar to the full-length protein [35]. Recently, it was demonstrated that the hydrophilic part of Tic110 actually contains four amphipathic transmembrane helices that are capable of forming

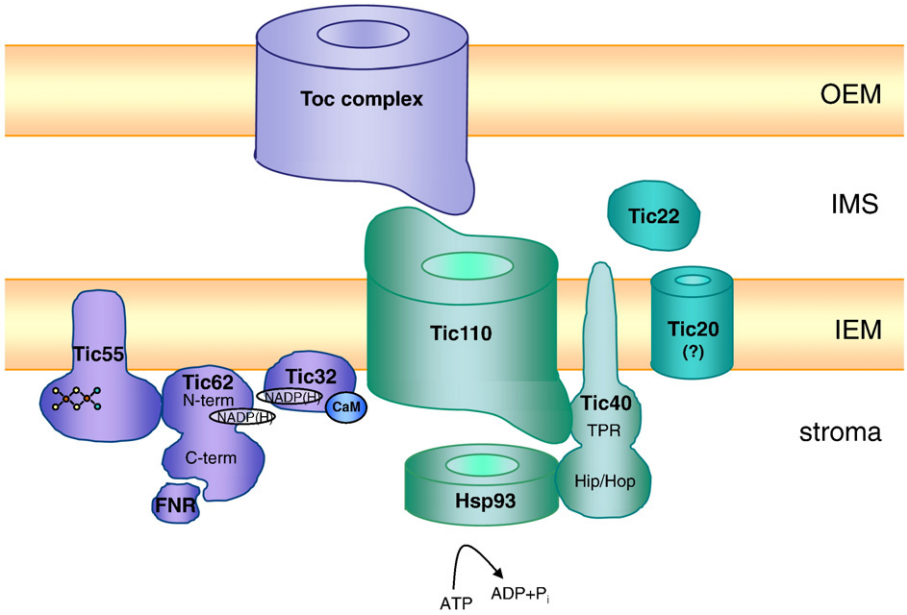

Fig. 2. Tic translocon machinery components. Tic110 and Tic20 were proposed to form a channel in the inner envelope membrane (IEM) of chloroplasts, although, the channel activity of Tic20 has not yet been shown. Tic110 is in contact with the Toc complex and also associated with Tic40 and the stromal chaperone Hsp93 forming the "motor complex" (coloured in green). Tic40 interacts with Tic110 via its TPR domain and with Hsp93 via the Hip/Hop-like domain. Hsp93 is depicted here as a hexameric ring, although it is not yet clear which oligomerization state is present in the import process. The only component located in the intermembrane space (IMS) is Tic22. Tic62, Tic55, and Tic32 form the redox regulon (coloured in purple). The NADP(H)- and calmodulin (CaM)-binding sites of Tic32 are indicated. Tic62 contains an NADP(H)-binding site at the N-terminus (N-term) and an FNR-interacting domain at the C-terminus (C-term). The Rieske-type iron–sulphur centre of Tic55 is symbolized.

a cation-selective channel [36]. According to this comprehensive model (taking into account all earlier findings), large domains face the stroma and could mediate the recruitment of stromal chaperones such as Hsp93 and Cpn60, providing the driving force for translocation and folding of preproteins [39–41]. On the other hand, contact with the Toc machinery and the incoming transit peptide is possible via loops facing the intermembrane space [34,41,42].

Tic110 is of eukaryotic origin, thus it is not present in any prokaryotic organisms including cyanobacteria [43,44]. In *A. thaliana*, Tic110 is encoded by a single-copy gene. It is expressed in flowers, leaf, stem, and root tissues indicating a role in all types of plastids [42,45]. Inaba et al. [41] observed that homozygous T-DNA insertion lines are embryo lethal, establishing that the Tic110 gene product is essential for growth. Reduction of the expression of AtTic110 results in a pale green phenotype, defect in plant growth, and strongly reduced amounts of thylakoid membranes and starch granules in chloroplasts. Furthermore, the authors reported a reduced content of nuclear-encoded chloroplastic proteins indicating a defect in protein import.

Several lines of evidence (the high abundance, its presence in different tissues, the embryo lethality of homozygous mutants, the channel activity, and the interaction with stromal chaperones) clearly pinpoint that Tic110 is the main pore of the Tic translocon. Additional channels might also exist to support the translocation or membrane insertion of certain preprotein subpopulations.

One candidate is **Tic20**, which is inserted into the inner envelope membrane via four predicted alpha-helical transmembrane domains [46]. Cross-linking experiments suggested that it is in close vicinity to an intermediate stage of import during protein translocation together with Tic22 [47,48]. Some structural relation was observed between Tic20 and the mitochondrial inner membrane translocon components Tim17 and Tim23 [44]. These Tim proteins have four transmembrane domains as well and form protein import channels in the inner mitochondrial membrane.

There are four Tic20 isoforms in *A. thaliana*, among which AtTic20-I shows the highest homology to Tic20 from pea [44]. Tic20 is present in all plant tissues and its expression is highest in rapidly growing tissues. It is much less abundant on the protein level in chloroplasts than Tic110 [49]. Comparing its amount to AtToc75-III, the main protein import channel in the outer envelope, it is approximately 10 times less, whereas the ratio between AtTic110 and AtToc75 is 1.4. The reduction of AtTic20-I expression causes a severe pale phenotype, growth defects, and deficiency in plastid function (e.g. smaller plastids, reduced thylakoids, decreased content of plastidic proteins, and altered import rates of preproteins) [50]. Interestingly, Tic20 is important for protein import and viability also in *Toxoplasma gondii* parasites, which contain a special type of plastids (called apicoplasts) surrounded by four membranes as a result of secondary endosymbiosis [51]. Additionally, Kikuchi et al. [52] described in *A. thaliana* and pea a protein complex with a size of about 1 Mda containing Tic20, small amounts of Tic21 (see below), and radiolabelled preprotein. Tic110 was not present in this complex, it was found separately in another, smaller molecular weight complex not containing the preprotein under the used conditions. According to the authors, the 1 megadalton complex could be an intermediate step in transferring the preprotein toward the Tic110-containing translocon. The above mentioned data suggest that Tic20 might also form a channel in the inner envelope, but direct experimental evidence for the channel activity, as well as the identification of its cargo protein is still missing.

Another potential Tic subunit, **Tic21**, was proposed by Teng et al. [53] as putative possible import channel. Reminiscent of Tic20, it is an integral inner envelope protein with four transmembrane helices. According to the authors, *attic21* null mutants show a severe pale phenotype and accumulate preproteins in the cytosol suggesting defect in protein import. By contrast, Duy et al. [54] characterized the same protein as an iron transporter (PIC1): The *atpic1* mutant plants are small and chlorotic as a result of impaired chloroplast development, but protein import into chloroplasts was described to be still functional. Furthermore, phytoferritin accumulates in the mutant chloroplasts. PIC1 has a high amino acid sequence identity (24%) with a cyanobacterial protein, encoded by the gene sll1656 in *Synecocystis*. To demonstrate the functional homology, *atpic1*/*attic21* knock out plants were successfully complemented with sll1656 [55]. Moreover, both proteins could complement the growth of iron-uptake yeast

mutants indicating a role in iron homeostasis and not in protein import [54].

Taken together, there are several candidates described so far that could form an import channel in the inner envelope. However, it is not clear yet whether they function independently of each other or they are part of different Tic complexes. They might act in different stages of development, in different tissues and cell types, or be responsible for various preprotein substrates, thereby increasing the complexity and specificity of the import process.

3. The motor complex

Early import studies already demonstrated that the energy for protein translocation through the inner envelope is provided by ATP hydrolysis, which is required for the function of an ATPase at the stromal side [56,57]. The cyanobacterial GroEL homolog and stromal chaperonin **Cpn60** was found to play a role in the import process in an ATP-dependent manner [39]. It was co-immunoprecipitated together with Tic110 during in vitro import experiments and this interaction was abolished in the presence of ATP. It is supposed to catalyse protein folding after translocation through the envelope membranes.

Another ATPase proposed to be involved in the import process is **Hsp93**: several cross-linking and co-immunoprecipitation experiments revealed a close proximity of Hsp93 to Tic110, supporting a role in protein import into chloroplasts [58–60]. Hsp93 (also called ClpC) is not only found to be associated to the import apparatus, but also to the Clp protease complex, originated from the cyanobacterial ancestor [61–64]. The protease consists of the proteolytic subunit of ClpP and the ATPase subunit of ClpC, the latter being responsible for substrate recognition, unfolding, and translocation into the proteolytic chamber.

Hsp93 is a member of the HSP100 molecular chaperone family. This family possesses unfolding capacity dependent on ATP hydrolysis. They form either hexameric rings (in the presence of nucleotides such as ATP or ADP) or migrate as smaller proteins (in the absence of nucleotides) [65]. Hsp93 has two distinct ATPase domains (AAA modules) and can be found in Gram-positive bacteria, cyanobacteria and plants. In *A. thaliana*, there are two nuclear-encoded Hsp93 genes present: AtHsp93-III and AtHsp93-V (also called ClpC2 and ClpC1, respectively). Both are highly identical on the amino acid sequence level (91%) but the expression level of AtHsp93-V is several-fold higher than that of AtHsp93-III [66]. The *hsp93-III* knock out plants show no phenotype, while *hsp93-V* plants are paler and retarded in growth in comparison to the wild type. Their chloroplasts develop less thylakoid membranes, contain a decreased amount of photosystem I and II proteins, and have reduced protein import efficiency [66–68]. The double knock out mutant of both Hsp93-coding genes is lethal, indicating that at least partially they are able to functionally substitute for each other in the single mutants [69].

According to the studies mentioned above, Hsp93 is supposed to have various functions in chloroplasts, one of them being protein import. However, it is still unclear how it exactly acts during the import process. **Tic40** was described as a co-chaperone, which can trigger the ATP hydrolysis of the "chaperone-motor" Hsp93 [70].

Tic40 consists of an N-terminal transmembrane domain, anchoring the protein into the inner envelope membrane, and a large hydrophilic C-terminal domain facing the stromal side [71]. The stromal part consists of one single TPR-like domain (see above) and a domain with similarities to Hip/Hop proteins (human *H*sp70 *i*nteracting *p*rotein/human *H*sp70 and Hsp90 *o*rganizing *p*rotein, in yeast called Sti1p) [60]. According to Chou et al. [70], the TPR domain of Tic40 is involved in the binding to Tic110, which is favoured in the presence of precursor proteins. Cross-linking experiments, yeast two hybrid and bimolecular fluorescence complementation assays revealed also a close proximity and interaction between Tic110 and Tic40 [60,71,72]. The second functional stromal part of Tic40 is the Hip/Hop domain, which mediates the interaction with Hsp93 [60,70]. In vitro experiments revealed that this interaction is stronger in the presence of ATP and non-hydrolyzable ATP than in the presence of ADP, indicating that Tic40 stimulates the ATPase activity of Hsp93.

Null mutants of *attic40* display a pale green phenotype, slower growth, and less grana stacks in the thylakoids than in wild type plants [60]. A reduced import rate into the chloroplasts from mutant plants causes decreased yield of nuclear-encoded proteins in the chloroplasts, whereas the binding of precursor proteins was not affected (according to the functional Toc complex). Chiu and Li [73] described an increased amount of Tic21 (PIC1), Tic40, and Tic110 soluble intermediates in the stroma in *attic40* null mutant chloroplasts during in vitro import experiments. They concluded that Tic40 is involved in the re-insertion of these proteins (and itself) into the envelope. However, Firlej-Kwoka et al. [74] described that the PIC1 import intermediate is already strongly membrane-bound (not extractable even with urea). This could indicate, that Tic40 is responsible for the insertion of the intermediate as well.

Different Tic40 constructs lacking either the transmembrane-, the TPR-, or Hip/Hop domain were not able to complement the *tic40* phenotype, indicating that the full-length protein is necessary for proper activity [72]. Interestingly, the phenotype of *tic40* knock outs could be complemented with a construct in which the Hip/Hop domain had been substituted by the corresponding domain of human Hip supporting a role for Tic40 as a co-chaperone.

Kovacheva et al. [66] described a similar phenotype for the heterozygous *attic110*, compared to homozygous *athsp93-V* and *attic40* mutants, and interestingly, all three double mutant combinations of these genes show the same phenotype without additive interactions (epistasis). Additionally, the interaction pattern with precursor and mature proteins during import experiments is highly similar for Tic110, Hsp93, and Tic40 [60]. The in vitro and in vivo data (cross-linking, co-immunoprecipitation, import studies, and mutant phenotypes) thus strongly suggest that these three components might act together, establishing the current model of the import channel (Tic110) associated with the motor complex consisting of a co-chaperone (Tic40) and a chaperone (Hsp93) for facilitating protein import into chloroplasts (Fig. 2).

4. Regulation of protein import across the inner envelope

Chloroplasts have to constantly adapt to the developmental and environmental conditions of the plant. Changing demands require adaptation of the protein complement and therefore protein import should possess the capacity to become regulated. This appears to occur via phosphorylation, GTP-hydrolysis (as both known for the Toc complex), the stromal redox status, and calcium/calmodulin signalling.

4.1. Redox regulation

Three proteins in the Tic translocon have domains which allow them to be involved in redox regulation. They contain either NADP(H)-binding sites (Tic62 and Tic32) or a Rieske-type iron–sulphur centre (Tic55) and constitute the so-called "redox regulon" of the complex (Fig. 3).

Tic62 was initially described to form a complex together with Tic110 and Tic55 [75]. Its N-terminus contains a highly conserved NADP(H)-binding site which is not only present in all oxyphototrophs but even in green sulphur bacteria [76]. Interestingly, the structure of the N-terminus changes in response to the presence of $NADP^+$ or NADPH [77]. An additional hydrophobic patch on the surface of the protein presumably allows the binding to lipids and to the inner envelope, while the central domain of the protein (amino acids 247–346 in PsTic62) is apparently involved in the binding to the Tic complex. The C-terminus of Tic62, in contrast to the N-terminus, is found only in seed plants and has a disordered structure [76,77].

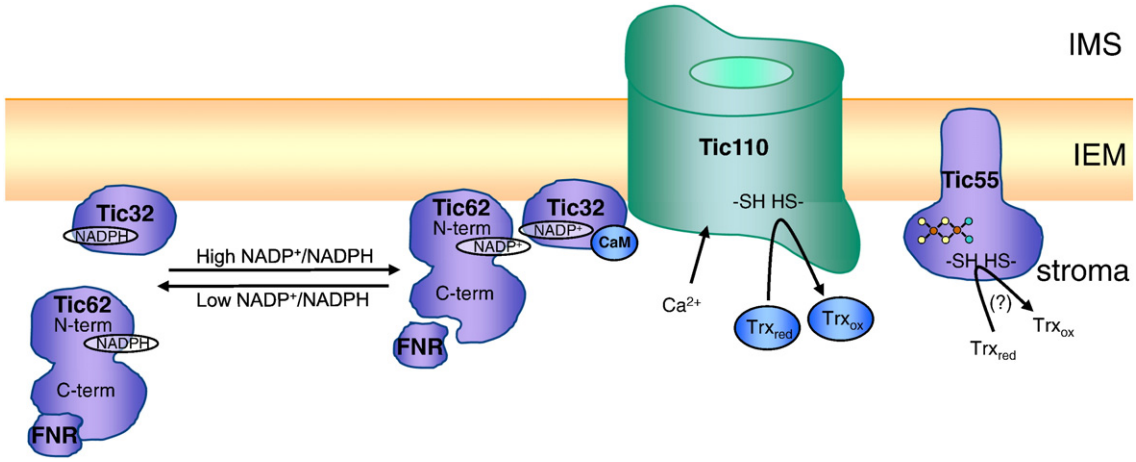

Fig. 3. Regulation of protein import into chloroplasts at the level of the inner envelope membrane (IEM). Metabolic redox regulation: depending on high or low $NADP^+$/NADPH ratio Tic62 is either more strongly attached to the Tic complex or is found more soluble in the chloroplast stroma. Furthermore, Tic62 binds stronger to FNR and Tic32 is dissociated from the Tic complex under reduced conditions. Thioredoxin (Trx) regulation: Tic110 and Tic55 are supposed to have Trx-regulated conserved Cys residues (only the redox active sulphydryl groups are indicated). Ca^{2+}/CaM regulation: Tic32 can bind to calmodulin (CaM) in the absence of NADPH. The channel activity of Tic110 can be altered in the presence of Ca^{2+}. IMS: intermembrane space.

This domain contains several Pro/Ser-rich repeats (the number is dependent on the species) involved in protein–protein interactions. It was demonstrated to specifically bind to ferredoxin-$NADP^+$-oxidoreductase (FNR). FNR catalyses the last step of photosynthetic electron transport in chloroplasts, delivering electrons from reduced ferredoxin to $NADP^+$. Via FNR, Tic62 could thus represent a link between photosynthesis and the protein import into chloroplasts. A recently identified protein, TROL, is supposed to tether FNR to the thylakoid membrane [78]. Its very strong interaction with FNR is enabled by a motif with high sequence similarities to the Tic62 FNR-binding site. The *attrol* mutants differ only slightly from the wild type, including lowered electron transport rates and increased non-photochemical quenching under high-light conditions, indicating a defect in electron transport. Whether or how the function of Tic62 and TROL might overlap remains to be investigated.

Tic62, such as Tic32, bears a NADP(H)-binding site at the N-terminus, belongs to the (extended) family of short-chain dehydrogenases, and was characterized as enzymatically active dehydrogenase [77,79]. For both proteins, dehydrogenase activity was only observed in the presence of NADPH and not of NADH and was dependent on a lipid environment. In addition, the localization of Tic62 was shown to be dependent on the chloroplastic $NADP^+$/NADPH ratio (Fig. 3). In an oxidized environment (high $NADP^+$/NADPH ratio) Tic62 is mostly membrane-bound and binds stronger to the Tic complex. Under reduced conditions (low $NADP^+$/NADPH ratio) it is located in the stroma and associates stronger with FNR. Accordingly, shuttling between the stroma and the inner envelope might allow Tic62 to transmit information about the chloroplast redox state generated mostly by the photosynthetic machinery (via FNR) to the translocon and thus enable redox regulation of protein import. This assumption led to the idea that Tic62 might act as a redox sensor for chloroplast import [75,77].

Tic32 was initially identified as interaction partner of the N-terminus of Tic110 [80]. Even though no transmembrane region was identified by computational analysis, it is tightly bound to the chloroplast inner envelope. As mentioned above, Tic32 has a conserved NADP(H)-biding site at the N-terminus and described to be an enzymatically active dehydrogenase [79]. It can dissociate from the Tic complex under reduced conditions (low $NADP^+$/NADPH ratio), but is presumably still membrane-bound (in contrast to Tic62), whereas under oxidized conditions (higher $NADP^+$/NADPH ratio) it is connected to the Tic complex. Tic32 was described to be an essential gene in *A. thaliana* [80], but later it was found not to be the case, the homozygous mutant plants are viable [81].

Hirohashi et al. [82] observed that the import efficiency of some precursors could differ under dark/light conditions: while some preproteins can be imported to the stroma in the light and in the dark as well, some others can be mis-sorted to the intermembrane space in the light, but imported efficiently in the dark. It raises the possibility, that in the light the high photosynthetic activity (and thereby the reduced environment via the decreased $NADP^+$/NADPH ratio) can affect the import efficiency. In a recent study, the connection between the metabolic redox state and import efficiency was characterized in isolated pea chloroplasts [83]. An increase of the $NADP^+$/NADPH ratio in the stroma was observed to favour the import efficiency of a subgroup of proteins e.g. involved in the Calvin–Benson cycle, metabolic pathways, light-harvesting, and photosynthetic electron transport. In contrast, this effect was not observed in *Physcomitrella patens* and *Chlamydomonas reinhardtii*. Intriguingly, in these organisms the Tic62 homologs contain only the NADP(H)-binding site and lack the FNR-binding domain. The fact that the regulation via the $NADP^+$/NADPH ratio is not present indicates that this might be a recent evolutionary regulatory pathway (possibly via Tic62) in seed plants.

Since the redox state does not affect the import of all tested preproteins using the general import pathway, the possibility of different Tic sub-complexes could be envisaged. According to Stengel et al. [83], the translocon complex could exist in two states: either as the Tic channel with motor complex (Tic110, Tic40, and Hsp93) or with an additionally associated redox regulon (additional Tic32, Tic62, and Tic55). In this case, the latter could mediate a redox regulated protein import. The association and dissociation of the redox regulon dependent on the $NADP^+$/NADPH ratio thus could permit a change in the translocation capacity.

Tic55, the third component of the redox regulon was identified by Caliebe et al. [84] in a complex with Tic110 and Hsp93 in blue-native gels. Two transmembrane helices at the C-terminus anchor the protein into the inner envelope, whereas the N-terminus consists of amphiphilic β-sheets oriented towards the stroma. Tic55 is a member of the small family of non-heme oxygenases defined by the presence of a Rieske-type iron–sulphur centre and a mononuclear iron-binding site (amino acids 142–175 and 248–264 in PsTic55, respectively). This family also includes chlorophyll a oxygenase (CAO), choline monooxygenase (CMO), pheophorbide a oxygenase (PAO) and a 52 kDa chloroplastic protein (Ptc52) [85]. Caliebe et al. [84] treated isolated chloroplasts with diethylpyrocarbonate (DEPC) (interfering Rieske centres by the modification of His residues) before and after the binding of the preprotein to chloroplast surface during in vitro import experiments. The authors described no effect on binding but the

inhibition of complete translocation and maturation, indicating that a functional His residue is involved in the translocation process at a later stage of import, which might belong to Tic55. However, recent in vitro import experiments with *attic55* mutants revealed that Tic55 is not the sole target of this reagent [86]. These mutants do not show any detectable phenotype and they have no difference in protein import efficiency into chloroplasts (neither in the presence of DEPC) compared to the wild type, suggesting that Tic55 is not essential under the applied conditions.

Rieske-type proteins often function in electron transfer chains, such as cytochromes in the thylakoid membrane of chloroplasts or in the respiratory chain in the mitochondrial inner membrane. This, together with the close proximity to Tic62, suggests a role of Tic55 in redox regulation of protein import and raises the intriguing possibility of an electron transfer chain being present at the inner envelope membrane involving Tic62, Tic32, and Tic55, although, until now there is no experimental evidence for the existence of an electron transfer chain between the Tic components. However, a number of electron carrier proteins have been detected in isolated spinach envelope, being part of a possible electron transfer chain containing flavins, iron–sulphur centres and semiquinones capable of taking electrons from NADPH [87]. Murata and Takahashi [88] identified a similar electron transfer chain in spinach envelope connected to the photosynthetic electron transfer at the site of plastoquinone. Although exact components are still not identified, one can speculate that it might be the Tic redox regulon in connection with FNR.

Some data suggest that the expression of the different Tic subunits might also adapt to the metabolic redox conditions. Vojta et al. [49] compared the gene expression and protein levels of Toc and Tic components in leaves and roots. They found a several-fold lower amount of Tic62 and Tic55 in roots than in leaves as compared to the level of Tic110 and Tic40. Similarly, Boij et al. [86] found very low levels of Tic55 expression in non-photosynthetic tissues. This indicates that the photosynthetic activity produces a more variable environment, which requires a more prominent presence of the redox regulon components (e. g. Tic62 and Tic55) enabling the metabolic redox regulation. Interestingly, the Tic subunits also show a different abundance in C_3 (*Pisum sativum*) and C_4 (*Zea mays*) plants [89]: envelope proteomics revealed that Tic55, together with Hsp93 and FNR1, have lower relative abundance in C_4 mesophyll envelopes, whereas Tic62 and Tic32 could not be identified, presumably according to their very low amount. The low abundance of these Tic components might be the result of the difference in the metabolic pathways (and thereby in the $NADP^+$/NADPH ratio) in C_3 and C_4 plants. All of these findings support the highly dynamic nature and complexity of the Tic translocon machinery adapting to different developmental and metabolic conditions.

Apart from regulation of protein import by the $NADP^+$/NADPH ratio another redox system, the thioredoxins, might also be involved in import redox regulation (Fig. 3). Thioredoxins are small multifunctional redox active proteins. By oxidation of conserved Cys residues they can reversibly reduce different types of proteins and thereby modulate their activity and/or conformation. In chloroplasts, four Trx types are known: Trx f and m are involved in the ferredoxin/thioredoxin system transmitting redox signals to target enzymes (for review see [90]). They had originally been described as activators of the Calvin–Benson cycle enzymes fructose-1,6-bisphosphatase and NADP-malate-dehydrogenase, respectively. Trx x and y primarily play a role not in regulation of enzyme activity, but in stress response. The increasing amount of identified Trx targets (e.g. components of the Calvin–Benson cycle, pentose phosphate cycle, the ATP synthase, light-harvesting antenna complex II, enzymes involved in lipid and starch biosynthesis, nitrogen and hydrogen metabolism, etc.) shows a central regulatory role of thioredoxins in chloroplasts. The involvement of thioredoxins in protein import into chloroplasts has also been suggested: Bartsch et al. [91] characterized Tic55 together with PAO and Ptc52 (two other members of the non-heme oxygenases family) as thioredoxin targets in the inner envelope membrane of chloroplasts. Tic110 was additionally found to be a Trx target, as it was shown to possess a redox active disulfide bridge, which can be reduced by stromal thioredoxins in vitro [36]. Interestingly, Tic110 was demonstrated to exist in a reduced state in isolated chloroplasts in the dark. It remains elusive under which physiological conditions (e.g. oxidative stress) the protein is oxidized and what the exact role of the disulfide bridge might be.

4.2. Calcium/calmodulin regulation

Calcium is a universal secondary messenger in eukaryotes and has several essential functions in the cell-signalling system. It can bind to various proteins, most importantly to calmodulins, which are then able to activate or deactivate other proteins, thereby transducing environmental signals [92].

Chloroplast protein import was also demonstrated to be influenced by the Ca^{2+}-signalling network of the plant cell (Fig. 3). Chigri et al. [93] reported an inhibition of import after the chloroplasts had been treated with the calmodulin inhibitor Ophiobolin A or the calcium ionophores A23187 and Ionomycin in a concentration-dependent manner. Only the import of proteins having a cleavable N-terminal transit peptide was affected, indicating that Ca^{2+} regulation affects the general import pathway. Tic32 was identified as a possible mediator of this Ca^{2+} regulation since it has a calmodulin-binding domain at its C-proximal end (amino acids 296–314 in PsTic32) and binds to calmodulin in a calcium-dependent manner [79]. Interestingly, this interaction is weakened in the presence of NADPH, indicating that the binding of the two molecules is mutually exclusive (Fig. 3).

Calmodulin has not been successfully identified so far in chloroplasts but there is evidence for the presence of a chloroplastic calmodulin, e.g. some chloroplast localized proteins such as PsaN, a component of photosystem I, the chaperonin Cpn10, and an ATPase (encoded by At3g56990) were found to be calmodulin-regulated as well [92,94]. Additionally, several chloroplast-located calmodulin-like proteins are described [92,93,95]. However, no direct connection between the calmodulin-like and the calmodulin-regulated proteins could be found. Interestingly, Tic110 was also observed to be regulated by Ca^{2+}, since the presence of Ca^{2+} has a profound effect on the channel activity of Tic110 in vitro [36].

In a recent study, it could be demonstrated that not just the chloroplast but also the mitochondrial protein import is influenced by the Ca^{2+}-signalling network. Kuhn et al. [96] described that plant mitochondrial import can be influenced by calcium ionophores as well as the calmodulin inhibitor Ophiobolin A. The target of this regulation is not yet known but seems to be localized in the inner membrane of mitochondria, similarly to chloroplasts.

5. Conclusion

Considering the increasing knowledge, the Tic translocon seems to be a highly complex and dynamic import machinery. Several modes of regulation, and various expression patterns dependent on species, tissues, or external stimuli provide the opportunity for a highly specialized and regulated import system. Due to the presence of different regulatory levels it seems to respond to environmental and metabolic conditions: First, sensing the $NADP^+$/NADPH ratio allows to adapt the import to the metabolic status of the organelle. Secondly, the involvement of thioredoxins renders protein transport responsive to light signals (referring to thioredoxins as the “eyes” of the chloroplast, [90]). And finally, Ca^{2+}/CaM regulation integrates the organelle into the signalling network of the cell. Focusing on the details of regulation possibilities will bring us closer to understand the exact functioning of the Tic translocon in the future.

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