Redox extends its regulatory reach to chloroplast protein import

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The import of chloroplast proteins synthesized in the cytosol of a plant cell is mediated by two multiprotein complexes or translocons located at the outer and inner membranes of the chloroplast envelope, respectively, TOC and TIC. These complexes integrate different signals to assure the timely transport of proteins into the chloroplast in accordance with the metabolic and developmental needs of the cell. The past few years have witnessed the emergence of redox as a regulator of the protein transport process. Here, we discuss evidence that the metabolic redox state of the chloroplast regulates the import of preproteins by altering either the activity or composition of participating transport components. It appears that, through these redox changes, chloroplasts communicate with other compartments of the plant cell.

Chloroplast proteome: need for coordination of different factories within the plant cell

Because the vast majority of chloroplast proteins are nuclear-encoded, plastid biogenesis relies on the efficient sorting of proteins imported from the cytosol and on their coordinated assembly with the smaller number of proteins synthesized within the organelle, for example photosystem multicomplexes or RuBisCO (Figure 1 and Box 1) [1–3]. Owing to changes in metabolism linked to the environment or stage of development, chloroplasts require adaptive responses. The proficient coordination of plastid function or stage of development, chloroplasts require adaptive responses. The proficient coordination of plastid function with activities of the plant cell requires rapid and efficient reciprocal communication. Plastid signaling is currently an area of intensive research that includes, among others, a coordination of plastid protein import and nuclear gene expression [4,5]. Herein, we discuss emerging evidence for the role of redox in regulating protein import as a function of the needs of the cell under changing conditions.

Original evidence for regulation of protein import by redox

Some years ago, the effect of light on protein import into chloroplasts was analyzed [6]. The authors performed in vitro import studies with precursors of the photosynthetic and non-photosynthetic isofoms of ferredoxin (Fdx) and ferredoxin–NADP reductase (FNR), FdxI/FdxIII and FNRI/FNRII, respectively. Interestingly, light had a strong, specific effect on import of the non-photosynthetic isofoms. Whereas the photosynthetic FdxI and FNRI isofoms were properly transported to the stroma with concomitant development of mature proteins after cleaving off the transit peptide in light and darkness, the non-photosynthetic FdxIII and FNRII precursors failed to enter the stroma and the preproteins accumulated in the chloroplast intermembrane space (IMS) in the light. The mistargeting to the IMS was not reversed when chloroplasts were transferred back to dark. However, if the precursor proteins were reisolated and imported into non-light treated chloroplasts, they were properly processed. The effect was ascribed to misfolding in the IMS and to the possibility that the translocation of certain precursor proteins across the envelope was regulated by light. The molecular determinants behind this observation have, however, remained elusive.

Other lines of evidence point to thiol-based redox as a regulator of protein import

In recent years, diverse mechanisms have been invoked to explain the import of proteins into chloroplasts. In an attempt to dissect the participating factors and characterize the energetic and protein requirements, in vitro import assays have been developed with the isolated organelle [7]. Following this approach and using the cysteine (Cys)-modifying reagent N-ethylmaleimide, earlier investigators concluded that functionally important Cys residues are involved in the formation of early import intermediates as well as in the import process itself [8,9]. These results were in line with independent research that showed a stimulation of import by dithiothreitol (DTT) or reduced glutathione added to the import reaction mixture [10]. A different approach demonstrated that the in vitro modification of Cys to yield a disulfide bridge inhibited the formation of early import intermediates and subsequent import [11]. Inhibition was relieved by DTT, thus demonstrating reversibility of the redox effect. Molecular analysis prompted the conclusion that members of the TOC machinery participated in the crosslinking reaction, namely the receptors Toc159 and Toc34 and the channel Toc75. These findings have been confirmed very recently [12]. The authors analyzed the effect on import not only of dithiol reagents, such as DTT, but also monothiols such as β-mercaptoethanol and reduced glutathione. It was concluded that, although both types of reducing agents enhanced protein import, dithiols were much more effective. Moreover, tris(2-carboxyethyl)phosphine (TCEP), a phosphine reagent that seems not to cross the chloroplast envelope and exert its effect on the outside, confirmed that
members of the outer translocon facing the cytosol contain Cys essential for protein import. It might well be that those subunits participate in a thiol–disulfide redox switch that governs import. By contrast, a comparison of the DTT and TCEP effects suggests that components of the inner envelope membrane might be affected.

During evolution, chloroplasts developed an intricate regulatory network based on the modification of Cys according to the changing redox status of the organelle [13]. A growing body of evidence indicates that certain components of TOC and TIC complexes have redox-active Cys with the potential to regulate protein function and
Review

Box 1. The general import pathway into chloroplasts

Several cytosolic factors have been invoked to facilitate the guidance of precursors to the TOC receptors and enhance the efficiency of translocation (Figure 1). The cytosolic Hsp70/14-3-3 complex might escort preproteins to the TOC GTPase receptors, Toc159 and Toc34 [57]. Another set of precursors seems to be guided by cytosolic Hsp90 that, after interaction with Toc34, delivers the precursor to Toc34 [58]. TOC GTPase receptors, whose GTPase activity is regulated by transit peptide binding, play a primary role in chloroplast targeting [3]. Although their relative contribution to preprotein recognition is a subject of intense debate, Toc159 and Toc34 probably heterodimerize in the docking stage with the transit peptide, contributing to the activation of membrane translocation. At the expense of ATP, the preprotein is transferred and transported through Toc75, a β-barrel transmembrane protein that constitutes the TOC channel [59]. Components of the chaperone system in the intermembrane space, composed of the DnaJ-like Toc12 and imaHsp70, seem to be recruited to the outer envelope by Toc64 [17,60]. Together with Tic22, a soluble TIC component in the IMS, they keep the preprotein in a competent unfolded state and coordinate the translocation to the TIC channel, probably Tic110 [19,61]. Concomitant with preprotein reception, Tic110 recruits components of the import motor (chaperone Hsp93 together with co-chaperone Tic40 and/or chaperone Hsp70) at the stromal side of the envelope [26,62]. Tic20 is also described as a protein-conducting channel. New experimental evidence obtained from a Tic20 homolog in apicocales suggests a regulatory role of Tic20 in protein import [63]. Functional complementation studies are in order to determine a conserved role of Tic20 in plants and apicocales. Another putative protein-conducting channel, Tic21, might compensate for Tic20 at different stages of leaf development [64]; an independent study has, however, classified PIC21/Tic21 as a permease [65]. Three redox-sensing auxiliary components transiently interact with the TIC core to modulate preprotein import according to the metabolic redox state of the chloroplast. Several modes of regulation affecting preprotein import efficiency have been identified: (i) modulation of the GTPase activity of the TOC receptors [86]; (ii) intra- or intermolecular covalent interactions in TOC components taking place under oxidizing conditions [11,12,15,17]; (iii) modification of TOC isomorph composition according to preprotein and/or plastid type [67–70]; (iv) variation in TIC protein composition as a reflection of chloroplast metabolic redox status [33,71]; and (v) modulation of activity of TIC components by regulatory Cys [19,28] (Figure 1).

Consequently, import into chloroplasts (Table 1) [14]. A recent study documents the ability of the single conserved Cys in the Toc34 family to undergo disulfide bridge formation under oxidizing conditions both in vitro and in organello [15]. The current view of receptor binding invokes a transient dimerization of receptors (Box 1), although stable dimers cannot be ruled out [16]. The formation of Toc159–Toc34 heterodimer via Cys crosslinking has been proposed but not demonstrated. Whether the receptors form regulatory disulfide bridges under physiological conditions remains to be established.

Toc12, a subunit of the TOC complex in the chloroplast intermembrane space that associates with Toc64 and Hsp70, might also be sensitive to Cys redox modification [17]. Structural prediction pointed to the proximity of two conserved Cys in the Toc12 family and point mutational analysis suggested the formation of an intramolecular disulfide bridge that regulates the chaperone activity of the protein. The authors suggested a functional relation of Toc12 with the mistargeting and arrest of certain preproteins in the IMS under light conditions [6]. However, biochemical evidence for Cys oxidation in Toc12 is still lacking as is information on redox fluctuation in the IMS as a consequence of light or changing metabolic status. To date, redox machineries equivalent to those catalyzing the oxidation of proteins in the periplasmic space of bacteria or the IMS of mitochondria have not been identified in the chloroplast envelope [18].

Evidence of a role for redox in regulating the preprotein conducting channel of the TIC complex of the inner membrane, Tic110, emerged in biochemical assays [19]. Experiments revealed a redox-active intramolecular disulfide bridge in Tic110, whose activity appears to be regulated by a stromal thioredoxin (Trx). Trx is a ubiquitous small disulfide protein that is well known to mediate redox changes in target proteins of both the chloroplast stroma and thylakoid membrane. A proteomic study suggested that an isoform of Trx is closely associated with the chloroplast inner envelope membrane [20]. Because the active disulfide of the Tic110 channel protein was found to be in the reduced state in chloroplasts isolated from darkened plants, it was concluded that regulation linked to redox status is particularly relevant during periods of oxidative stress as chloroplasts adapt to changes in the environment.

During import, Tic110 recruits the stromal motor complex, composed of soluble Hsp93 and membrane-anchored Tic40, at the envelope. Hsp93 (also known as ClpC) acts as a stromal chaperone in protein import. Interestingly, the activity of the cyanobacterial homolog of Hsp93 has also been linked to Trx [21]. The redox active disulfide(s) is yet to be identified. Earlier genetic studies showed that the expression of cyanobacterial hsp93 is modulated by the redox state of the cell [22]. By contrast, in plants, transcript levels of the gene increased in short-day ntre rosette leaves of knockout plants lacking chloroplast NADP-thioredoxin reductase with a built-in Trx [23]. Even if the chloroplast and cyanobacterial Hsp93 proteins function in different pathways and are not equally regulated, they are both likely to act as chaperones [24]. Sequence analysis of cyanobacterial Hsp93 revealed a single conserved Cys residue in this chaperone family, suggesting that, if linked to Trx, the regulatory disulfide would be inter- rather than intramolecular [14]. Structural homology modeling of the plant Hsp93 isoform is consistent with the formation of an intramolecular disulfide bridge due to the presence of a second conserved Cys. According to the proposed model, the conserved Cys would be located in the nucleotide-binding motif, suggesting that the disulfide bridge could have either a structural role or function in regulating ATPase activity or in forming the ternary Hsp93–Tic40–Tic110 complex. Further experiments are needed to confirm the formation of a disulfide bridge in chloroplast Hsp93 and to evaluate its functional significance.

It is noteworthy that a mixed disulfide bridge developed between Tic110 and the co-chaperone, Tic40, under oxidizing conditions [25]. Tic40 is a membrane protein with two stroma-exposed hydrophilic domains, one of which preferably interacts with Tic110 and the other with Hsp93 [26]. However, the only conserved Cys in Tic40 capable of oxidative crosslinking with Tic110 is found in the most C-terminal stromal domain, which is indeed predicted to associate with Hsp93 [27]. Refinements in the association analyses in the ternary complex between the import motor
### Table 1. Redox properties of proteins involved in chloroplast protein transport (Figure 1)

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Function</th>
<th>Redox element</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toc75</td>
<td>Channel-forming protein at the outer envelope membrane.</td>
<td>Participates in disulfide crosslinking with TOC GTPases under oxidizing environment. Contains conserved cysteines at the N-terminal POTRA domain and at the beginning of the so-called motif 3, an active membrane element in transport.</td>
<td>[11,12,14]</td>
</tr>
<tr>
<td>Tic159 and Tic34</td>
<td>GTPase receptors of the TOC complex.</td>
<td>Involved in intermolecular disulfide bridge formation. Contains one conserved cysteine in the GTPase domain involved in dimer formation.</td>
<td>[11,12,14,15]</td>
</tr>
<tr>
<td>Toc12</td>
<td>DnaJ-like co-chaperone at the IMS.</td>
<td>Contains a CxGxxC motif at the DnaJ domain involved in a functional important intramolecular disulfide bridge.</td>
<td>[17]</td>
</tr>
<tr>
<td>Tic110</td>
<td>Channel-forming protein at the inner envelope membrane.</td>
<td>Forms intramolecular disulfide bridge under oxidizing conditions. Activity probably regulated by stromal Trx.</td>
<td>[19]</td>
</tr>
<tr>
<td>Hsp93</td>
<td>Chaperone of the TIC protein import motor.</td>
<td>The cyanobacterial ortholog has been linked to Trx. Computational analysis points to conserved cysteine residues in plant Hsp93 with potential for intramolecular disulfide bridge formation.</td>
<td>[14,21]</td>
</tr>
<tr>
<td>Tic62</td>
<td>Redox sensor of the TIC complex.</td>
<td>Bimodular protein. The N-terminal domain belongs to the short-chain dehydrogenase family and contains an NADP(H)-binding motif; the C-terminal domain binds specifically and interacts with FNR. Dynamic binding to the TIC complex according to the metabolic redox state of the chloroplast.</td>
<td>[30,33,34]</td>
</tr>
<tr>
<td>Tic32</td>
<td>Redox sensor of the TIC complex.</td>
<td>Bimodular protein. The N-terminal domain belongs to the short-chain dehydrogenase family and contains an NADP(H)-binding motif; the C-terminal tail interacts specifically with stromal calmodulin. Dynamic binding to the TIC complex according to the metabolic redox state of the chloroplast.</td>
<td>[31,33]</td>
</tr>
<tr>
<td>Tic55</td>
<td>Redox sensor of the TIC complex.</td>
<td>A membrane protein that exposes to the stroma a Rieske domain, a catalytic domain that coordinates a non-heme Fe(II) and a hydrophilic CxxC domain of unknown function. Activity probably regulated by stromal Trx.</td>
<td>[28,29]</td>
</tr>
</tbody>
</table>

Hsp93–Tic40 and the Tic110 protein channel are obviously in order.

Tic55, another member of the TIC complex, has also recently been linked to Trx [28]. Tic55 is a membrane protein with three hydrophilic domains exposed to the stroma: a Rieske domain, a catalytic domain with a mononuclear non-heme Fe(II) active site and a domain of unknown function that carries a conserved CxxC motif [29]. The specific contribution of Tic55 to preprotein import remains elusive. It has been proposed that the protein might regulate import via its function in electron transfer or as a redox sensor. In a recent study, the authors captured Tic55 from an inner envelope preparation on an affinity column using a mutated Trx-f as bait [28]. Sequence analysis showed that Tic55 contains several conserved Cys that could form an oxidant-induced disulfide bridge in the regulation of protein import [14,28].

**Effects of the relative NADPH/NADP ratio on protein import: the TIC redox translocon**

Molecular studies on the import machinery of the chloroplast envelope revealed that the Tic62 and Tic32 subunits contain binding motifs for redox-active cofactors that possibly affect protein import [30,31] (Table 1). Together with the Rieske protein Tic55, these components have been proposed to form the “redox regulon” of the TIC complex [32].

Tic32 was found to be extrinsically attached to the inner envelope membrane and to contain a functional NADP(H)-binding domain characteristic of the family of short-chain dehydrogenases [33,34]. Biochemical analysis demonstrated that its interaction with the translocon is regulated by the chloroplast NADPH/NADP ratio: more oxidizing conditions (lower NADPH/NADP ratio) favor association of Tic32 with the TIC translocon, whereas more reducing conditions (higher NADPH/NADP ratio) have the opposite effect and lower the affinity of the Tic32 subunits for the core of the TIC complex. Tic32 members also contain a C-terminal extension that specifically binds chloroplast calmodulin (CaM) in a manner that is enhanced under oxidizing conditions. Moreover, in vitro protein import experiments have demonstrated that the translocation of certain substrates is impeded by CaM inhibitors or calcium ionophores [35]. Considering that the calcium level in chloroplasts fluctuates in a light/dark-dependent manner, it seems plausible that metabolic factors such as CaM modulate the interaction of Tic32 with the core of the TIC complex.

The interaction of Tic62, another short-chain dehydrogenase family member, with the TIC core complex has also been found to depend on the metabolic NADPH/NADP ratio. Like Tic32, the affinity of Tic62 for the TIC complex is influenced by chloroplast redox status. Association is increased with lower NADPH/NADP ratios, conditions under which Tic62 shows higher affinity for FNR, a second specific interaction partner [33,34]. Furthermore, Tic62 can reversibly shuttle between the envelope membrane and the stroma as a function of the relative NADPH/NADP ratio. Under more reducing conditions, Tic62 is partially released to the stroma.
The molecular features of Tic62 and Tic32 were recently put in the context of protein import as a reflection of their modification by the chloroplast NADPH/NADP ratio [12]. These authors observed that perturbing this ratio altered the import efficiency of some but not all proteins that follow the general import pathway (Box 1). The import of other proteins taken up by import mechanisms apparently unrelated to TOC and TIC was also not affected by this ratio. It was suggested that the interaction of Tic62 and Tic32 with TIC might account for the observed effect. The recruitment of the redox regulated subunits to the translocon might, therefore, result in different TIC subcomplexes in which Tic62 and Tic32 couple the redox state of chloroplasts to nucleotide binding and thereby alter import.

Despite the apparent importance of redox in regulating preprotein import into chloroplasts, genetic studies have revealed that none of the genes encoding proteins of the “redox regulon” seems essential for plant viability under standard conditions. The function of the encoded proteins might, therefore, lie either in fine-tuning of transport of several substrates or in enabling chloroplasts to adjust to a change in environmental conditions [36–38].

Redox control transport and folding in the thylakoidal lumen

Recently, it was found that 40% of the proteins residing in the lumen are linked to Trx or a related redox-active protein, thus underscoring the importance of redox-linked regulation in this compartment [39–42]. It is of interest that lumenal proteins have two options for import, the cpTat and cpSec pathways, both adapted from bacteria [43] (Figure 1). In the cpTat pathway, prefolded proteins are translocated into the lumen after entering and achieving their correct conformation in the stroma. By contrast, cpSec translocates unfolded units in an ATP-dependent manner with folding occurring after translocation. The SecA subunit has been recently linked to Trx in cyanobacteria [21]. Although further biochemical experiments are in order, it appears that the disulfide bridge in SecA is redox-active, thus offering a role for redox in regulating thylakoid protein transport from the stromal side in both plants and cyanobacteria [14]. Unlike their stromal counterparts, redox-active proteins of the lumen examined so far are active when oxidized [44]. However, there appears to be no correlation between redox activity of transported proteins and mode of import. For example, although both are redox-active, FKBP13 and OE33 are translocated by different pathways (cpTat and cpSec, respectively) [45,46]. Based on current knowledge, it seems that selection of a particular translocation pathway is determined by chaperone and, possibly, by redox requirements of proteins harboring active Cys residues. The situation is reminiscent of the sulphydryl oxidation system functional in the IMS of mitochondria and the periplasmic space of bacteria [18]. In both cases, specialized machineries catalyze the oxidative folding of proteins via a disulfide relay system involving coordination between soluble regulatory components and the transmembrane redox transfer system. However, in contrast to these systems, little is known about the redox properties and regulatory mechanisms active in the lumen and new possibilities are just beginning to emerge [14,47–49].

Looking to the future

Recent observations have added new insight to the complex and highly regulated process of preprotein import into chloroplasts. On one hand, change in the organization of the TOC GTPase receptor isoforms results in different TOC complexes, each apparently with affinity for a particular class of preproteins. On the other hand, the oxidative cross-linking of subunits of the outer envelope translocon influences the transport efficiency of a subset of preproteins when in vitro protein import experiments are performed in isolated chloroplasts. The TIC machinery is also subjected to dynamic changes in composition and activity through its response to regulatory redox signals from the chloroplast. Thus, although the relative NADPH/NADP ratio typically remains stable in chloroplasts due to photosynthesis during the day and to the oxidative pentose phosphate pathway at night, the equilibrium might be significantly altered as a result of changes in metabolism, particularly during adaptation to oxidative stress conditions. Current evidence suggests that the activity of several TIC subunits is linked to redox by way of Trx, particularly in response to oxidative events.

The unveiling of a role for redox in regulating and coordinating chloroplast protein import raises new questions. For example, biochemical mechanisms that link metabolic redox state of the cell to the reversible association of redox-sensing subunits with the TIC complex are still poorly understood. Further research is also needed to elucidate specific redox modifications in translocon subunits and their relation to import activity. The endogenous substrates for the redox sensor components await identification as do participants that transmit redox information to the translocon machinery. According to present evidence, Trx could communicate a redox signal to Tic110, Hsp93 and Tic55, probably during environmental adaptation. The Cys residues functional in these and other participating proteins need to be identified and characterized. A related unknown concern is the consequence of redox modification on protein activity. Questions similar to these apply to the TOC subunits that contain redox-active Cys, some exposed to the cytosol. Finally, a major area for future development concerns the mechanism by which redox-active proteins are transported and folded following their entrance into the thylakoid lumen via the cpTat and cpSec (Box 2) [43].

Acknowledgements

M.B. acknowledges support from the Spanish National Research Council (2009401204). Support from the Deutsche Forschungsgemeinschaft (SFB 594 and CIPSM) to J.S. is gratefully acknowledged. B.B.B. gratefully acknowledges receipt of a Research Award from the Alexander von Humboldt Foundation and ongoing support from the California Agricultural Experiment Station.

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