Characterisation of components and mechanisms involved in redox-regulation of protein import into chloroplasts

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Summary

The vast majority of chloroplast proteins is encoded in the nucleus and thus has to be posttranslationally imported into the organelle, a process that is facilitated by two multimeric protein machineries, the Toc and Tic complexes (**t**ranslocon at the **o**uter/**i**nner envelope of **c**hloroplasts). Regulation of protein import, *e.g.* by redox signals, is a crucial step to adapt the protein content to the biochemical requirements of the organelle. In particular, one subunit of the Tic complex, Tic62, has been proposed as a redox sensor, whose possible function is to regulate protein import by sensing and reacting to the redox state of the organelle. To elucidate a potential redox regulation of protein import, structural features, redox-dependent properties and the evolutional origin of Tic62 were investigated. The results show that Tic62 consists of two very different modules: the N-terminal part was found to be mainly α-helical and possesses dehydrogenase activity *in vitro*. It is furthermore an evolutionary ancient domain, as it is highly conserved in all photosynthetic organisms from flowering plants to cyanobacteria and even green sulfur bacteria. In contrast to this, the C-terminus is largely disordered and interacts specifically with ferredoxin-$NADP^+$ oxidoreductase (FNR), a key enzyme in photosynthetic electron transfer reactions. Moreover, this domain was found to exist only in flowering plants, and thus the full-length Tic62 protein seems to be one of the evolutionary youngest Tic components. The results of this study make also clear that Tic62 is a target of redox regulation itself, as its localization and interaction properties depend on the metabolic redox state: oxidized conditions lead to fast membrane binding and interaction with the Tic complex, whereas reduced conditions cause solubilization of Tic62 into the stroma and increased interaction with FNR. This novel shuttling behaviour indicates a dynamic composition of the Tic complex. The $NADP^+$/NADPH ratio was furthermore found to be able to influence the import efficiency of many precursor proteins. Interestingly, the import of not all preproteins depends on the stromal redox state. Hence it was proposed that not a single stable Tic translocon exists, but several Tic subcomplexes with different subunit compositions, which might mediate the import of different precursor groups in a redox-dependent or -independent fashion. Another redox signal that was analyzed in regard to an impact on protein import is the reversible reduction of disulfide bridges, which was found to affect the channel and receptor proteins of the Toc complex. The import of all proteins that use the Toc translocon for entering the chloroplast was shown to be influenced by disulfide bridge formation. Thus it can be concluded that a variety of redox signals, acting both on the Toc and Tic complexes, are able to influence chloroplast protein import.

Zusammenfassung

Die Mehrzahl der in den Chloroplasten lokalisierten Proteine ist im Nukleus kodiert und muss folglich posttranslational in das Organell importiert werden. Dieser Prozess wird von zwei multimeren Proteinkomplexen bewerkstelligt, den so genannten Toc und Tic Komplexen (**t**ranslocon at the **o**uter/**i**nner envelope of **c**hloroplasts). Die Regulation des Proteinimports, z.B. durch Redoxsignale, ist äußerst wichtig um den Proteingehalt an die biochemischen Anforderungen des Chloroplasten anzupassen. Eine der Untereinheiten des Tic Komplexes, Tic62, wurde als potentielles Sensorprotein vorgeschlagen, welches den Proteinimport regulieren könnte, indem es den Redoxzustand in den Chloroplasten misst und diese Information an den Tic Komplex weitergibt. Um Einblicke in die Redoxregulation des Proteinimports zu erhalten, wurden in dieser Arbeit die Struktur, die redox-abhängigen Eigenschaften und der evolutionäre Ursprung von Tic62 untersucht. Die Ergebnisse legen nahe, dass Tic62 aus zwei sehr verschiedenen Modulen besteht: der N-terminale Teil besitzt *in vitro* Dehydrogenaseaktivität und besteht vor allem aus α-Helixes. Außerdem handelt es sich bei diesem Teil um eine evolutionär sehr alte Domäne, da sie in allen photosynthetischen Organismen von Blütenpflanzen bis zu Cyanobakterien und sogar grünen Schwefelbakterien stark konserviert ist. Im Gegensatz dazu weist der C-Terminus eine ungeordnete Struktur auf und vermittelt die spezifische Interaktion mit der Ferredoxin-$NADP^+$-Oxidoreduktase (FNR), welches ein Schlüsselenzym in photosynthetischen Elektronentransportprozessen darstellt. Außerdem wurde diese Domäne ausschließlich in Blütenpflanzen gefunden, deshalb scheint das vollständige Tic62 Protein eine der evolutionär jüngsten Tic Komponenten zu sein. Außerdem konnte in der vorliegenden Arbeit gezeigt werden, dass Tic62 selber durch Redoxsignale reguliert wird, da seine Lokalisierung und Interaktionseigenschaften vom metabolischen Redoxzustand abhängig sind: oxidierte Bedingungen führen zu einer schnellen Bindung von Tic62 an Membranen und zu einer verstärkten Interaktion mit dem Tic Komplex, wohingegen reduzierte Bedingungen eine bevorzugte stromale Lokalisierung von Tic62 auslösen, was mit einer stärkeren Interaktion mit der FNR einhergeht. Dieses neuartige Verhalten einer Tic Komponente impliziert eine dynamische Zusammensetzung des Tic Komplexes. Es konnte zusätzlich gezeigt werden, dass der stromale Redoxzustand in der Lage ist, die Importeffizienz von vielen Vorstufenproteinen zu beeinflussen. Interessanterweise ist nicht der Import von allen Vorstufenproteinen vom metabolischen Redoxzustand abhängig. Daher ist es möglich, dass nicht ein einziger Tic Komplex, sondern verschiedene Subkomplexe mit unterschiedlichen Zusammensetzungen existieren, welche den Import von

verschiedenen Klassen von Vorstufenproteinen redox-abhängig oder -unabhängig regulieren können. Die reversible Reduktion von Disulfidbrücken in den Kanal- und Rezeptorproteinen des Toc Komplexes wurde als weiteres Redoxsignal untersucht, das ebenfalls den Proteinimport regulieren kann. Es konnte gezeigt werden, dass der Import aller Vorstufenproteine, welche den Toc Komplex zum Eintritt in den Chloroplasten benötigen, von der Disulfidbrückenbildung beeinflusst wird. Somit kann die Schlussfolgerung gezogen werden, dass eine Reihe von unterschiedlichen Redoxsignalen, die sowohl am Toc als auch am Tic Komplex wirken, in der Lage sind, den Proteinimport zu beeinflussen.

Introduction

The origin of chloroplasts and a general description of protein import

Plastids are a diverse group of organelles found in all plants and algae. Members of this family include amyloplasts, which store large amounts of starch, chromoplasts, which accumulate red, orange or yellow carotinoid pigments and elaioplasts, which store lipids (for reviews see Neuhaus & Emes, 2000; Lopez-Juez & Pyke, 2005). Nevertheless, the phylogenetically most original plastid is the chloroplast, which is also the most prominent and well-studied plastid type, as its main function is the conversion of light into chemical energy by photosynthesis. Additionally, they play essential roles in many other biosynthetic pathways such as fatty acid biosynthesis, nitrite and sulphate reduction and amino acid biosynthesis (for reviews see Lopez-Juez & Pyke, 2005, Nelson & Ben-Shem 2004).

It is a widely accepted theory that chloroplasts derived from an endosymbiotic event in which an early eukaryotic cell (that already contained a likewise endosymbiotic mitochondria) engulfed a cyanobacterial prokaryote (Margulis 1970). As it is believed that this so-called primary endosymbiosis occurred only once in plant evolution, all plastids share a common ancestry. During subsequent evolution, this new organelle lost its autonomy by transferring most of its genes (>90%) to the host cell nucleus (for review see Leister 2005). Thus, the endosymbiont became dependent on protein import from the surrounding cell, although it still maintained a rudimentary but essential genetic system for DNA replication, transcription and translation. Dependent on the species, the chloroplast genome encodes only ~ 50-250 proteins, whereas the proteome is thought to consist of ~3500 polypeptides (for reviews see Gould *et al.* 2008, Leister 2003). Consequently, the vast majority of chloroplast proteins is synthesized on cytosolic ribosomes and has to be posttranslationally targeted to and imported across the two envelope membranes into the organelle. Most of these proteins contain an N-terminal targeting signal called transit peptide (TP), which is necessary for targeting of the precursor to the organelle, recognition by receptor proteins and translocation through both membranes. These TPs do not display significant similarities at the level of primary sequence or secondary structures and furthermore vary in length between 20-150 amino acids (von Heijne *et al.* 1989). However, all TPs are enriched in hydroxylated amino acids such as threonine and serine, are hydrophobic at the extreme N-terminus and show an overall positive charge (for reviews see Cline 2000, Bruce 2000). Targeting to the chloroplast is supported by binding of the preproteins to molecular chaperones like Hsp90 and Hsp70, the latter forming a

"guidance complex" together with 14-3-3 proteins that have been reported to increase the efficiency of protein import *in vitro* (May & Soll 2000, Zhang & Glaser 2002, Qbadou *et al.* 2006). The binding to chaperones in the cytosol is necessary to maintain an import competent conformation of the precursor. Translocation itself is mediated by multiprotein complexes in the outer (OE) and inner envelope membrane (IE): the Toc (**t**ranslocon at the **o**uter envelope of **c**hloroplasts) and the Tic (**t**ranslocon at the **i**nner envelope of **c**hloroplasts) complexes (for reviews see Inaba & Schnell 2008, Jarvis 2008, Stengel *et al.* 2007, Benz *et al.* 2008). Translocation via this pathway is an energy-consuming process (Figure 1), requiring both ATP and GTP, which defines three different import steps (Perry & Keegstra 1994, Kouranov & Schnell 1997): in the first energy-independent binding step, the transit peptide binds reversibly to the Toc receptor components. In the second irreversible step, the precursor is inserted into the Toc channel and makes contact with components of the Tic complex, which requires both GTP as well as low ATP concentrations. In the last step, where high ATP concentrations are needed, the precursor is completely translocated into the stroma (Figure 1). After import, the TP is cleaved off by the stromal processing peptidase (SPP), resulting in the mature form of the protein (VanderVere *et al.* 1995) and folding into the active conformation by stromal chaperones or subsequent sorting into further chloroplast compartments (for reviews see Keegstra & Cline 1999, Gutensohn *et al.* 2006, Jarvis 2008).

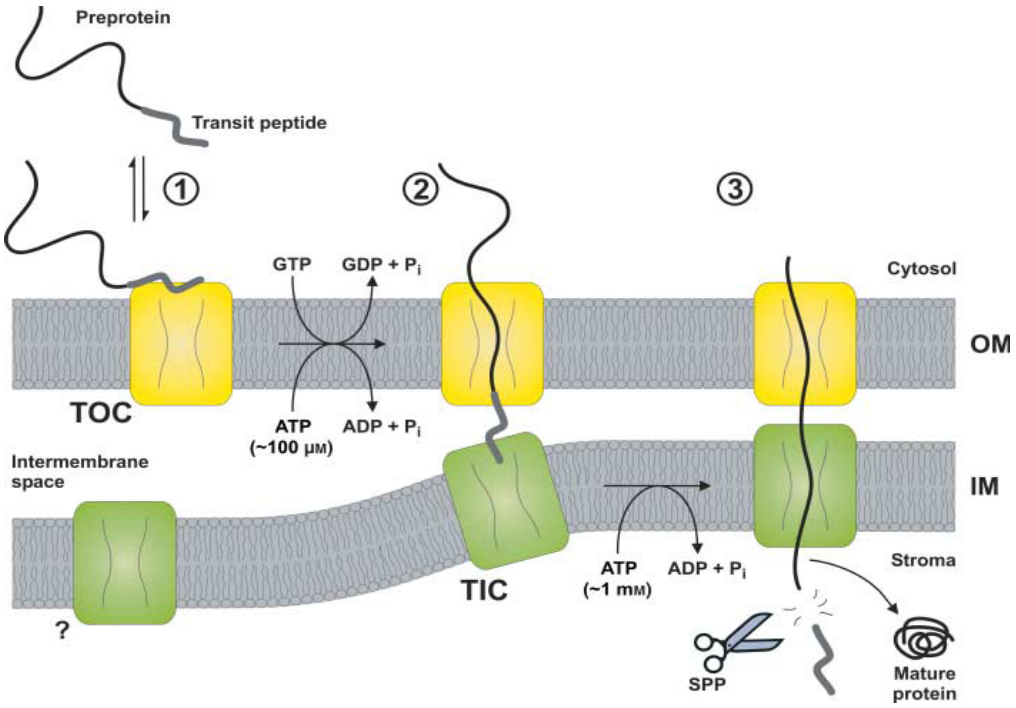

Figure 1 (Jarvis 2008): Protein import into chloroplasts can be divided into three distinct steps: (1) the preprotein binds reversibly to the Toc receptors independent of energy, (2) the precursor is inserted across the outer envelope and interacts with the Tic complex, which requires GTP and low amounts of ATP. (3) In the final ATP-dependent step, translocation across both membranes is completed and the TP is cleaved in the stroma by the stromal processing peptidase (SPP), followed by folding of the preprotein in its final conformation.

The Toc complex

The Toc complex in the outer chloroplast envelope is responsible for precursor binding and translocation across the outer membrane, and consists of five subunits (Figure 2): two GTPases, Toc159 and Toc34, function as receptor components and are able to bind and hydrolyze GTP (Kessler *et al.* 1994, for review see Jarvis 2008). It was proposed that TP interaction with Toc34 occurs upstream of those with Toc159. The affinity of Toc34 to the precursor is greatly increased in the GTP-bound state (Becker *et al.* 2004a). An exchange of GTP to GDP leads to a complex with lower affinity for the preprotein than in the GTP-bound form. The GTP/GDP exchange seems to be caused by either heterodimerisation of Toc34 and Toc159 or by stimulation of the intrinsic GTPase activity by the preprotein. Therefore, the preprotein dissociates from Toc34 and is transferred to Toc159-GTP. Toc159 is proposed to act as a GTP-driven motor pushing the preproteins through the translocation channel (Schleiff *et al.* 2003). Toc64 was described to function as another receptor, as it was found to interact with Hsp90-associated preproteins (Qbadou *et al.* 2006). This is in contrast to preprotein recognition by Toc34 and Toc159 that seem to be responsible for binding of precursor proteins being in a complex with Hsp70 and 14-3-3 proteins. Toc64 contains three TPR (tetratricopeptide repeat) motifs in the C-terminus that are proposed to be involved in protein-protein interactions and mediate the association of proteins with molecular chaperones (for review see Frydman & Hohfeld 1997).

Toc75 has been shown to constitute the translocation pore of the Toc complex (Schnell *et al.* 1994) and belongs to the Omp85 superfamily. It possesses a β-barrel structure of 16-18 transmembrane strands and is functionally equivalent to Tom40, the translocation channel in the outer membrane of mitochondria (Schnell *et al.* 1994, Hinnah *et al.* 1997, Hinnah *et al.* 2002). The members of the Omp85 family mediate a variety of protein transport processes in bacteria and mitochondria and also function in the biogenesis of β-barrel proteins (Voulhoux *et al.* 2003, for review see Reumann & Keegstra 1999). Furthermore, Toc75, Toc159 and Toc34 were found to form the Toc core complex, a molecular machine of approximately 550 kDa, with a Toc75/Toc34/Toc159 molecular stoichiometry of 4:4:1 (Schleiff *et al.* 2003). Toc12, the smallest subunit of the Toc complex contains a J-domain for interaction with imHsp70 (Becker *et al.* 2004b) and is located in the intermembrane space (IMS), where it seems to constitute a complex together with Toc64 and the Tic component Tic22. This complex seems to be required for the coordination of the Toc and Tic translocons during import, as it is thought that preproteins pass both complexes simultaneously.

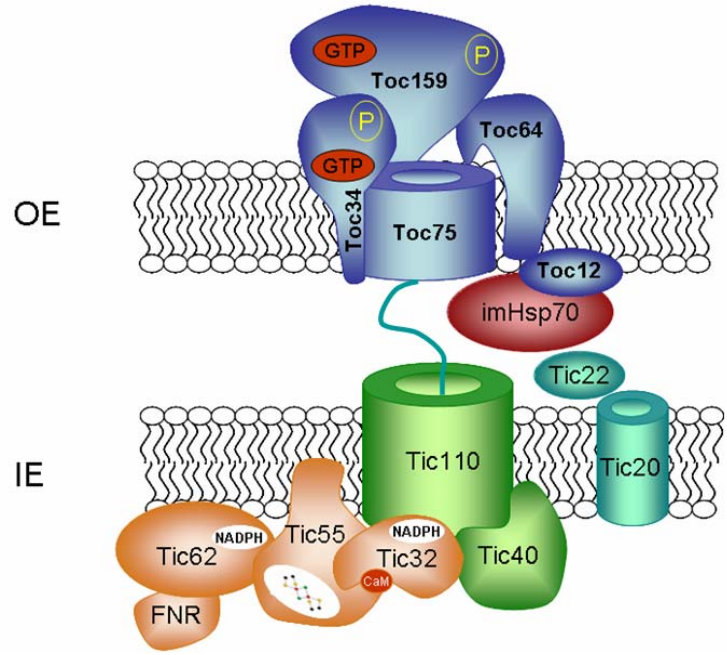

Figure 2 (adapted from Stengel *et al.* 2007): Model of the Toc and Tic complexes that contains all the components identified to date. Preproteins are recognized by receptor components of the Toc complex (Toc34, Toc159 or Toc64), followed by translocation through the Toc75 channel. In the intermembrane space, Toc12, imHsp70 and Tic22 interact with the incoming preproteins and mediate the interaction of the Toc and Tic complexes. Tic110 constitutes the channel of the inner envelope membrane and can also recruit stromal chaperones, probably with the help of the co-chaperone Tic40. Tic32, Tic55 and Tic62 are involved in redox regulation of the import process. Tic20 forms another putative translocation channel.

The Tic complex

The Tic complex is a multimeric protein machinery that facilitates translocation of proteins through the inner envelope, a process that is independent of GTP but requires ATP, probably due to the involvement of stromal chaperones (Figure 2). The Tic complex is thought to consist of at least seven proteins (for reviews see Benz *et al.* 2008, Jarvis 2008): Tic110 is the most abundant Tic protein and forms the central translocation channel (Heins *et al.* 2002, Balsera *et al.* 2009). It is involved in many steps of the translocation process, including assembly of Toc-Tic "supercomplexes" (Akita *et al.* 1997, Nielsen *et al.* 1997), preprotein recognition (Inaba *et al.* 2003), translocation, and folding of imported precursor proteins in the stroma (Kessler & Blobel 1996) by the recruitment of chaperones (Cpn60 and ClpC/Hsp93) to the import site. These chaperones may not only function in folding of imported proteins, but are also part of an import motor that fixes the preprotein in the stroma and thus prevents retrograde movement. Tic110 consists of two very different domains, an N-terminus with two hydrophobic transmembrane helices, which anchor the protein in the membrane, and a long rather hydrophilic C-terminal domain. This C-terminus contains four amphipathic helices that are able to insert into the membrane to constitute a cation-selective

ion channel, which is sensitive to chloroplast TPs (Heins *et al.* 2002, Balsera *et al.* 2009). Furthermore, Tic110 was recently found to contain several conserved cysteine residues and to exist in an oxidized and a reduced form *in vitro*. Stromal thioredoxins (Trx) were described to mediate the reduction of Tic110, thus the channel activity might be influenced by the Trx system (Balsera *et al.* 2009, see below).

Tic40 contains one transmembrane helix in the N-terminus that anchors the protein in the inner envelope, while the C-terminus faces the stroma (Stahl *et al.* 1999). This part includes a TPR-motif and a domain that shows homologies to the co-chaperones Hip (Hsp70-interacting protein) and Hop (Hsp70 and Hsp90 organising protein). Thus, it was proposed that Tic40 acts as a co-chaperone in the Tic motor complex, together with Tic110 and Hsp93/ClpC. The only Tic component localised in the IMS is Tic22, which belongs to the IMS-complex described above and seems to be only loosely bound to the inner envelope. The smallest member of the Tic complex, Tic20, contains four transmembrane helices and shows structural homologies to the mitochondrial inner membrane channel proteins Tim17 and Tim23. It is thought to form another putative translocation channel, although no channel activity has been published yet.

Furthermore, three Tic proteins were proposed to act as potential redox-active subunits (the "redox regulon"): Tic62, Tic32 and Tic55. Tic55 contains a Rieske-type [2Fe-2S] cluster and a mononuclear iron binding site (Caliebe *et al.* 1997). It is predicted to contain two transmembrane helices with the majority of the protein being exposed to the stroma. Database analysis revealed homology of Tic55 to the CAO/PAO-like oxygenases (pheophorbide a oxygenase, chlorophyll a oxygenase), which act *e.g.* in chlorophyll biogenesis or oxygen dependent degradation pathways, and also to bacterial aromatic ring-hydroxylating dioxygenases. Rieske proteins are often involved in electron transfer reactions, *e.g.* in the cytochromes present in the respiratory chain of mitochondria or in the photosynthetic complexes in the thylakoids of chloroplasts, but so far it has not been studied whether Tic55 acts as an oxygenase *in vitro* or *in vivo*. Recently, Tic55, which contains a conserved pair of cysteines (CXXC), has been identified as target of stromal Trx, but the physiological significance of this interaction has not yet been investigated (Bartsch *et al.* 2008). Tic32 belongs to the large family of short-chain dehydrogenases and was originally identified as an interaction partner of the N-terminal part of Tic110 (Hörmann *et al.* 2004). It exhibits all characteristic motifs of short-chain dehydrogenases, like a β-sheet stabilizing motif in the

central domain and an active site in the C-terminus. Additionally, it contains a NADP(H)-binding site and possesses dehydrogenase activity *in vitro*. Although not having a transmembrane domain, Tic32 is found to be firmly attached to the inner envelope. Moreover, it was identified as the predominant calmodulin (CaM)-binding protein of the inner membrane, suggesting that both, redox regulation (via NADPH) and calcium regulation (via CaM) of chloroplast protein import could be mediated by Tic32 (Chigri *et al.* 2006).

The third potential redox-regulatory protein is Tic62 (Küchler *et al.* 2002), which possesses two redox-related features: in the N-terminus, a pyridine-nucleotide binding site can be found (similar to Tic32) that groups Tic62 into the extended family of short-chain dehydrogenases. The C-terminal part contains several unique repeats rich in serine (Ser) and proline (Pro) residues, which mediate the specific binding of Tic62 to the flavoenzyme FNR (ferredoxin-$NADP^+$-oxidoreductase). In higher plants, two distinct forms of FNR can be distinguished: a root (FNR-R1 and FNR-R2 in *Arabidopsis thaliana*) and a leaf form (FNR-L1 and FNR-L2 in *Arabidopsis*). The root isoforms are predominantly found in non-photosynthetic plastids, where they mediate electron flow from NADPH to ferredoxin (Fd). Reduced Fd subsequently donates electrons *e.g.* for nitrite reduction (Oji *et al.*, 1985, for review see Neuhaus & Emes, 2000). The best known function of FNR is mediated by the leaf form, which catalyzes the final step of the photosynthetic electron transport chain by transferring electrons from Fd to $NADP^+$, generating NADPH for several metabolic processes, as *e.g.* carbon fixation (Ceccarelli *et al.* 2004, Lintala *et al.* 2007). The FNR leaf form has also been implicated in cyclic electron transfer around photosystem I, which generates a proton gradient across the thylakoid membrane, resulting in ATP production without accumulation of reducing equivalents (Johnson *et al.* 2005). Thus, FNR plays a crucial role in photosynthesis and chloroplast redox-controlled metabolism. Because of the specific interaction of Tic62 with this important enzyme and because of its ability to bind pyridine nucleotides, it was proposed that Tic62 acts as a redox-regulatory protein that is able to sense and react to the redox state of the organelle. The interaction of Tic62 and FNR could furthermore provide a link between the redox state of the organelle (influenced mainly by photosynthesis) and redox-controlled protein import. However, only limited information was available about the function, structure and potential redox-dependent properties of Tic62. The interaction mode of Tic62 with FNR and with the Tic complex remained unclear as well. Thus these questions were addressed in the present study.

Alternative import pathways

The import of nuclear-encoded preproteins into the chloroplast using the Toc and Tic machineries is called the general import pathway. Additionally, alternative import pathways have been described and some of them have been discussed controversially (Figure 3). Several outer envelope proteins, including most Toc proteins and *e.g.* OEP7, OEP16, OEP21, OEP24 and OEP37, do not contain a cleavable transit peptide and seem to insert into the outer envelope membrane directly and spontaneously from the cytosolic side, independent of proteinaceous components (for reviews see Stengel *et al.* 2007, Jarvis 2008). As they lack an N-terminal TP, the targeting information is located in the mature part of these proteins. The same is true for an inner envelope protein called chloroplast envelope quinone oxidoreductase homologue (ceQORH), where an internal sequence and not the N-terminus was found to be required for translocation (Miras *et al.* 2002). However, some proteinaceous components are necessary for ceQORH import, although translocation was shown to be independent of the standard Toc/Tic machinery (Miras *et al.* 2007). Another example for an inner envelope protein without a cleavable TP is Tic32, which also seems to import without the assistance of the Toc and Tic complexes (Nada & Soll 2002). In contrast to ceQORH, the extreme N-terminus was found to be essential for chloroplast targeting, although it is not cleaved after translocation. An additional import pathway was suggested for proteins that contain a signal peptide for the secretory pathway but were nevertheless found to be localized in the chloroplast. These proteins (*e.g.* the carbonic anhydrase 1, CAH1) first seem to use their signal peptide to enter the endoplasmatic reticulum (ER), before they are subsequently transported to the Golgi apparatus and finally to the chloroplast with the help of the vesicle transport system (Villarejo *et al.* 2005, for review see Radhamony & Theg 2006). Substrates of this pathway are then proposed to be released into the intermembrane space by vesicle fusion with the outer membrane, from where they finally reach the stroma via either an unknown transporter in the inner envelope, via the Tic complex or via vesicle budding from the membrane itself. However, there is no proof for any of these possibilities so far. The participation of the ER and the Golgi suggests that this secretory pathway is required for the import of glycosylated proteins into the chloroplast, although the significance of glycosylation is not yet clear and needs further investigation.

Although only a few proteins have been described to use alternative translocation pathways so far, it is nevertheless conceivable that several other proteins also import independently of the Toc/Tic complexes. Studies of the chloroplast proteome revealed many nucleus-encoded

proteins that are located in the chloroplast but do not contain predicted cleavable targeting sequences (Kleffmann *et al.* 2004). Furthermore, several proteins with predicted ER signal peptides were found in the organelle proteome, which can not all be explained by mere contamination during chloroplast preparation. Thus, it seems likely that more proteins using alternative import pathways will be discovered in the future.

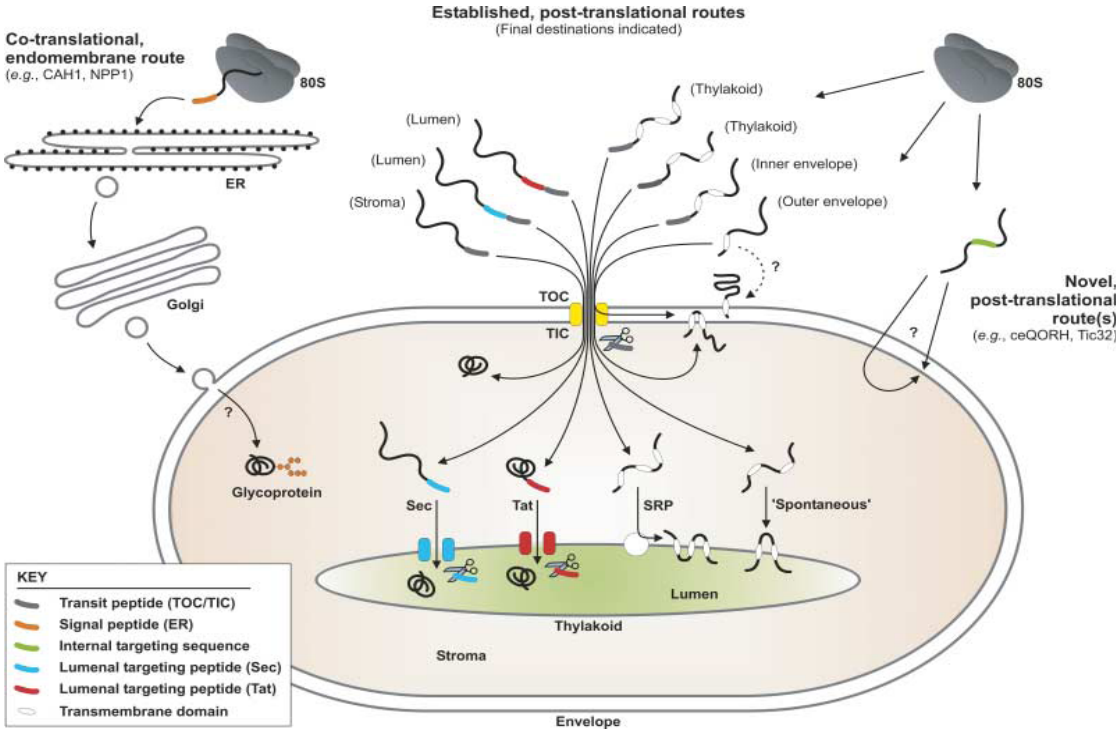

Figure 3 (Jarvis 2008): Overview of the protein targeting pathways to and inside the chloroplast. The majority of preproteins are translocated into the chloroplast by the general import pathway using the Toc and Tic complexes. Proteins destined for the stroma fold into their native conformation, otherwise they can be further targeted to the thylakoids (by the Sec, Tat, SRP or spontaneous insertion pathway). Many OE proteins do not contain a TP, and some of them seem to insert into the OE without the help of proteinaceous components. IE proteins may either use stop-transfer signals that cause lateral exit from the Tic channel into the membrane, or they are completely translocated into the stroma followed by insertion to the IE. Several alternative import pathways independent of the Toc and Tic complexes have been described, including novel uncharacterized pathways for the import of some inner envelope proteins as well as vesicle transfer to the chloroplast via the ER and the Golgi system.

Evolution of the import machineries

The evolutionary ancestors of plastids, the cyanobacteria, lack a protein import system. Since the majority of genes were transferred from the organelle to the host nucleus during evolution, a mechanism for protein translocation back into the chloroplast had to be established. Parts of the import machinery are thought to be derived from proteins present in the cyanobacterial endosymbiont, whereas other components seem to have been recruited from the eukaryotic host. The only component of the Toc complex that seems to have cyanobacterial origin is Toc75 (Bölter *et al.* 1998) as it belongs to the large prokaryotic Omp85 family of β-barrel

proteins. Neither the GTPase proteins (Toc159, Toc34) nor Toc64 seem to have cyanobacterial homologues, indicating a eukaryotic origin for these receptor components (Reumann *et al.* 2005). The J-domain containing protein Toc12 has no obvious relationship to cyanobacteria as well and has only been identified in *Pisum sativum* to date.

Three components of the Tic complex are clearly derived from cyanobacterial homologues: Tic20, Tic22 and Tic55 (Fulda *et al.* 2002, for review see Reumann & Keegstra 1999). Although some putative Tic32 sequences have been found in cyanobacteria, it is unclear if these are functional orthologs of Tic32, as only the NADPH binding site, but not the CaM-binding domain is conserved (Kalanon & McFadden 2008). As CaM-binding is a eukaryotic trait not found in prokaryotes, it seems likely that this function of Tic32 was added after endosymbiosis occurred. Thus, the Tic32 homologues might only be classical short-chain dehydrogenases, but a function of Tic32 in protein import seems to have been developed only recently in evolution. Tic110 and Tic40 seem to be of eukaryotic origin, since no obvious homologues have been found in cyanobacteria. Interestingly, Tic62 was found to be only partly of cyanobacterial origin: the N-terminal domain is related to prokaryotic nucleoside-diphosphate sugar epimerases, whereas the FNR-binding C-terminal domain is proposed to have been added rather late in evolution (Reumann *et al.* 2005). In contrast to the available information about Tic62 from flowering plants like *Pisum sativum*, no full-length homologues could be identified in the non-vascular plant *Physcomitrella patens* yet (Hoffmann & Theg 2003), suggesting that phylogenetic analyses of Tic62 might be of interest and were performed in the present study.

It seems likely that the development of an import apparatus originated from a primitive translocation machinery present in the ancestor of plastids, which already contained channels of the secretory machineries (*e.g.* Sec, Tat). The first Tic elements of an early translocon were probably the protein conducting channels Toc75 and Tic20, which were derived from the cyanobacterial endosymbiont (Reumann *et al.* 2005). These components could have been associated with the other proteins of cyanobacterial origin (Tic22, Tic55). The next proteins that were recruited to the developing translocation complex probably included the channel Tic110 that first had to be acquired from the host cell and the receptor components Toc159 and Toc34, followed by the co-chaperone Tic40. The remaining translocon subunits Toc64 and Toc12, and also the regulatory subunits Tic32 and Tic62 seem to belong to the evolutionary youngest members of the import machineries (Reumann *et al.* 2005).

Regulation pathways of protein import at the outer an inner envelope

To ensure that the amount of proteins present in the chloroplast correlates to the demand of the organelle at any given time, protein import should be tightly regulated. Possible regulatory mechanisms have to be coordinated between the host cell and the chloroplast and include regulation of transcription, mRNA stability, translation, as well as posttranslational modifications and protein turnover inside the organelle. Additionally, regulation of the amount of incoming preproteins via the Tic and Toc translocons is another striking possibility, which offers the advantage of a fast adaptation to the metabolic requirements of the chloroplast. The Tic and Toc complexes are therefore perfectly localized to impose a regulatory control over incoming preproteins. Generally, regulation of protein import can not only take place at both translocons but may already occur in the cytosol. For import regulation at a cytosolic stage, phosphorylation of the TP has been demonstrated (Waegemann & Soll 1996; Martin *et al.* 2006), which was shown to have an influence on the rate of translocation, since Toc34 has a higher affinity for phosphorylated than for non-phosphorylated precursors. Regulation of protein import at the stage of the Toc complex can also be achieved by phosphorylation of the receptor components Toc34 and Toc159 and by GTP/GDP binding (for review see Kessler & Schnell, 2004), as binding of GTP increases the affinity of Toc34 for the TP (Becker *et al.* 2004a). Phosphorylation of Toc34 was shown to cause inactivation of the receptor, as phosphorylated Toc34 is not able to bind to preproteins or to GTP.

Regarding the Tic complex, a Ca^{2+}/CaM mediated regulation has been postulated. Ca^{2+} is a common secondary messenger that regulates many biochemical processes by binding to Ca^{2+} sensing proteins such as CaM. Upon Ca^{2+} binding, CaM proteins change their affinities to target proteins which are thereby activated leading to further downstream responses (for reviews see Berridge *et al.* 2000, Yang & Poovaiah 2003). Import experiments revealed a response of protein import for the Ca^{2+}/CaM system, and Tic32 was identified as the predominant CaM-binding protein of the inner envelope (Chigri *et al.* 2005, Chigri *et al.* 2006). Thus it was proposed that the import might be regulated by Ca^{2+} signals, with CaM being the mediator, and with Tic32 as sensor protein. Finally, several studies indicated a redox-controlled regulation of protein import: the $NADP^{+}$/NADPH ratio was shown to have a clear influence on the composition of the Tic complex, as at least parts of the redox regulon (Tic62 and Tic32) seem to dissociate from Tic110 in the presence of NADPH (Chigri *et al.* 2006). Moreover, the participation of thiol-groups (Pilon *et al.* 1992, Seedorf & Soll 1995) and of stromal Trx (Bartsch *et al.* 2008, Balsera *et al.* 2009) in import regulation was

proposed (see below). However, the mode of redox regulation of the import process and the Toc and Tic components involved remains enigmatic, thus one aim of this study was to investigate potential redox regulation pathways of protein import in more detail.

Redox signals in the chloroplast

Redox regulation plays an important role in almost all metabolic or developmental processes in the chloroplast. Redox energy mainly originates at the photosynthetic electron transport chain. However, electron transfer processes are not only restricted to the photochemical reactions but are also important in a regulatory manner. Redox signals are known to be involved in regulation of many enzymes from *e.g.* the Calvin-Benson cycle or other key metabolic pathways (for review see Buchanan & Balmer 2005). They can additionally act as part of a signalling cascade from the organelle to the nucleus to regulate transcription (Fey *et al.* 2005). Trx were identified to play crucial roles in these regulation processes, as they are able to activate (or deactivate) chloroplast enzymes by reversible reduction of disulfide bonds (Figure 4). Disulfide bonds can be inter- or intramolecular and may be purely structural to stabilize the protein by maintaining its tertiary structure, or regulatory leading to catalytic changes in the target proteins.

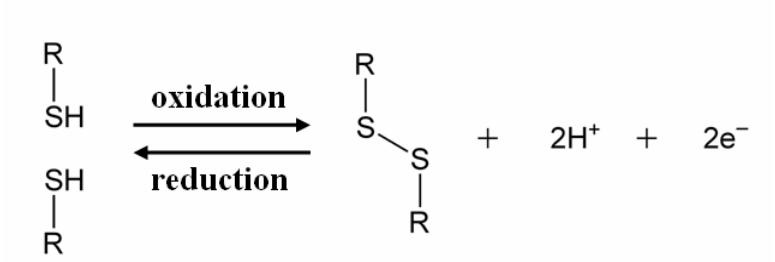

Figure 4: Oxidation and reduction of disulfide bridges. The thiol groups (SH) of cysteine residues can be reversely oxidized to form disulfides (S-S). Reduction of disulfides can be achieved by *e.g.* thioredoxins.

The thioredoxin system

Trx are small ubiquitous proteins with a characteristic structural motif (four α-helices surrounding a five-strand β-sheet) which are present in all living cells. Unlike in bacteria and animals, a large number of Trx have been identified in plants, *e.g.* 22 in *Arabidopsis thaliana* (for review see Hisabori *et al.* 2007). They can be grouped into six subfamilies (types f, m, x, y, o and h), of which Trx f, m, x and y are localized in chloroplasts, Trx o in mitochondria and Trx h in several cell compartments (cytosol, mitochondria and ER). The groups h, f and o are of eukaryotic origin, whereas groups m, x, y and z have prokaryotic ancestors. Recently, a

novel Trx type s has been identified in *Medicago truncatula*, which is localized in the ER and seems to be dedicated to symbiotic processes (Alkhalfioui *et al.* 2008). Initially described as a regulator for fructose-1,6-bisphosphatase (FBPase) and other enzymes of the Calvin-Benson cycle, many more Trx targets have been identified in the last years by proteomic approaches (for review see Montrichard *et al.* 2008). By now, these targets seem to be involved in almost every major cellular process like *e.g.* transcription, translation or carbon assimilation (Figure 5). Interestingly, no sequence or structural similarity has been observed in the Trx target proteins despite conserved cysteine residues often arranged in CXXC motifs. Regulation of plastidial proteins by redox modulation seems to be a common trait, in contrast to cytosolic enzymes, which are mostly regulated by the protein-kinase/protein-phosphatase system.

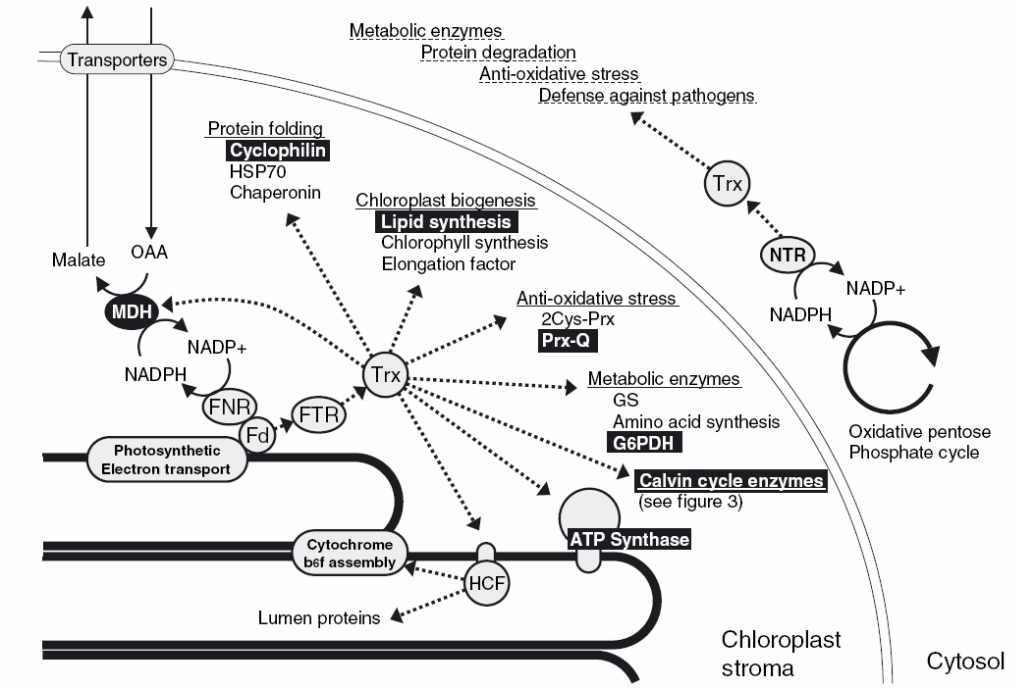

Figure 5 (Hisabori *et al.* 2007): Trx networks in the chloroplast and the cytosol. Trxs can be reduced either by ferredoxins (catalyzed by the ferredoxin–thioredoxin reductase, FTR), which takes place in the chloroplasts, or by NADPH (catalyzed by the $NADP^+$–thioredoxin reductase, NTR) in the cytosol. Pathways and target proteins (*e.g.* the malate dehydrogenase, MDH) of the Trx system are depicted. Dotted arrows show the electron flow.

Trx can be reduced by either ferredoxin (Fd) or NADPH, and are thus members of two different redox systems: the Fd–Trx system (FTS) of oxygenic photosynthesis located in chloroplasts and cyanobacteria, and the $NADP^+$–Trx system (NTS) localized both in the cytosol and mitochondria (Figure 6). The FTS contains Fd, Fd–Trx reductase (FTR) and Trxs f, m, x and y. In this system, Fd is reduced in the light by the photosynthetic electron flow which donates the electrons to Trx via the FTR. Subsequently, reduction of target proteins via thiol-disulfide exchange occurs. Re-oxidation of the system in the dark is most likely

achieved by transfer of the electrons to molecular oxygen (or oxidized Trx, glutathion or reactive oxygen species). Thus, the chloroplast exists in a reductive state in the light which changes to a more oxidative state in the dark. In contrast to the FTS, the NTS is comprised of $NADP^+$–Trx reductase (NTR), which transfers electrons from NADPH to Trx h in the cytoplasm or to Trxs h and o in mitochondria, followed again by reduction of the target proteins. In most cases, thiol reduction in the targets leads to enzyme activation, but deactivation of some enzymes (*e.g.* the glucose-6-phosphate dehydrogenase, Scheibe & Andersen 1981) has also been reported.

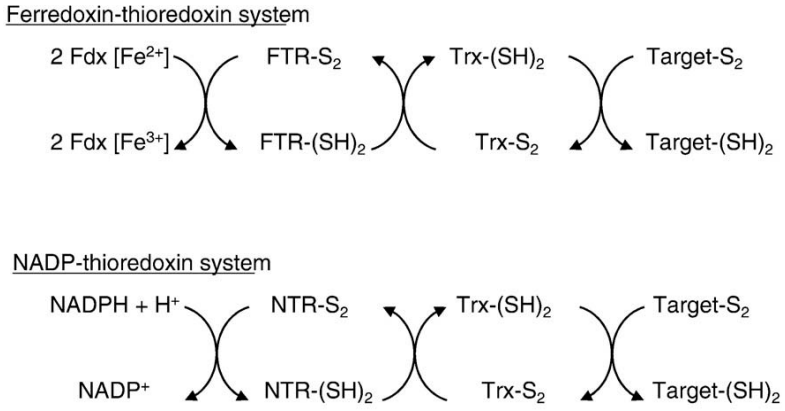

Figure 6 (Montrichard *et al.* 2008)**:** The Fd–Trx (FTS) and the $NADP^+$–Trx system (NTS). In both systems, electrons are transferred from either Fd or NADPH to target enzymes via Trx reduction. The electron transfer to Trx is mediated by the enzymes Fd–Trx reductase (FTR) or $NADP^+$–Trx reductase (NTR), respectively. Electron flow is achieved by thiol-disulfide exchange.

Redox-mediated regulation of protein import

Since the Tic complex contains three proteins with potential redox-active motifs, and because of the association of the photosynthetic enzyme FNR with Tic62, a redox regulation of protein import was proposed already several years ago (Caliebe *et al.* 1997, Küchler *et al.* 2002). Additionally, as the demand of the chloroplast has to be sensed inside the organelle, redox regulation at the Tic complex could be a fast way to react to metabolic changes. Thus, the potential redox-active subunits in the Tic complex, including Tic62, seem to be ideal candidates to sense and react to the metabolic stromal redox state. More recently, additional studies suggested even more pathways to be involved in redox-regulated import control.

There are two implications for Trx-mediated import regulation at the level of the Tic complex: firstly, using Trx-affinity purification methods with barley inner envelope, Tic55 was recently identified as Trx target. The protein contains a redox-active CXXC motif that

might mediate this binding and was found to interact both with Trx f and m (Bartsch *et al.* 2008). Secondly, the central Tic channel Tic110 was described to contain six conserved cysteines which form at least one redox-active disulfide bridge. Oxidation of Tic110 was found to be reversible (Balsera *et al.* 2009), suggesting that Tic110 exists in at least two redox states *in vitro*. Interestingly, Tic110 is present in a reduced form in chloroplasts maintained in the dark, which is in contrast to the generally oxidized state of the chloroplast stroma under this condition. To investigate a more physiological role of the Tic110 redox states, the reduction of Tic110 by Trx was analyzed. Indeed, both Trx f and m are able to reduce Tic110 *in vitro*, and thus it was concluded that Tic110 is a potential target of Trx in the stroma of chloroplasts. Although these data suggest a role of Trx in dark/light regulation of protein translocation, the physiological relevance of this finding remained to be investigated.

Beside the possible participation of Trx in regulating protein import, there are also indications that the $NADP^+$/NADPH ratio might be important for import regulation at the inner envelope. Previous studies reported differential import behaviour of the non-photosynthetic FdIII and FNRII from maize in light compared to dark (Hirohashi *et al.* 2001): both proteins were found to be mis-sorted to the IMS as an unprocessed precursor form in the light, which differed from import in the dark, where they were targeted into the stroma and processed to the mature form. This is in contrast to translocation of the photosynthetic FdI, which was efficiently imported into the stroma both in the light and in the dark, suggesting that the translocation of precursor proteins across the envelope membranes of chloroplasts may involve substrate-dependent, light-regulated mechanisms. These light/dark changes influence the redox state of protein thiol groups but also have a distinct impact on the stromal redox system (*e.g.* the $NADP^+$/NADPH ratio), thus making the latter a potential import regulatory signal. The $NADP^+$/NADPH ratio also seems to play a role in mediating the composition of the Tic complex. The interactions of the two redox regulon members Tic32 and Tic62 with Tic110 were found to be clearly diminished if isolated inner envelope vesicles were treated with NADPH. Obviously, these components dissociate from the Tic complex under reduced conditions, in contrast to oxidized conditions (presence of $NADP^+$), where they were found to be part of the Tic complex (Chigri *et al.* 2006). It was proposed that the chloroplast might contain several Tic subcomplexes with different subunit compositions, which are highly dynamic dependent on the metabolic chloroplast redox state. The association with and dissociation from Tic110 of Tic32 and Tic62 is thought to alter the activity or the substrate specificity of the channel in a yet unknown fashion.

Redox regulation of protein import might not only be mediated by signals acting on the Tic complex. Also the Toc complex was proposed to be redox-regulated, in this case mediated by the formation of disulfide bridges. It was demonstrated 20 years ago that the thiol modifying chemical N-ethylmaleimide (NEM) lowered the affinity for preprotein binding to pea chloroplasts and inhibited import (Friedman & Keegstra 1989). Another study found that preincubation of chloroplasts with the reducing agent DTT caused a clear import stimulation of Fd, an effect that was shown to influence the chloroplast import machinery and not the translation product (Pilon *et al.* 1992). Moreover, oxidizing compounds such as $CuCl_2$ were described to strongly inhibit preprotein binding and protein import (Seedorf & Soll 1995), which was found to be reversible by the addition of DTT after the $CuCl_2$ treatment. All these data implied the participation of thiol groups, which seem to play an essential role in this process. However it remained unknown which translocon proteins are the targets of this regulation and if the observed effects were only true for some preproteins, or if a general mechanism of import regulation by reduction of certain components exists.

Aim of this study

The biochemical needs of the organelle have to be carefully adapted to developmental or environmental changes, which often affect the rate of photosynthesis and thus the stromal redox state. The described findings and observations furthermore make a redox regulation of protein import into chloroplasts very likely. Since Tic62 was identified as an ideal candidate to sense and react to the redox state of the organelle, this protein was investigated in detail in the present study. The analyses focused on (i) the evolution of Tic62, since it was described that the protein seems to be only partially of prokaryotic origin. (ii) The function of Tic62, including its redox-related properties as well as the mode of interaction with the Tic complex and the FNR, and an influence of the chloroplast redox state on translocon behaviour and composition. (iii) The structure of Tic62, which is proposed to give further insight into its functional features. Additionally, the influence of different potential redox signals on import regulation was investigated, with a focus on translocation components and precursor proteins involved. This should give a first idea about the mode of redox-related import regulation both at the Toc and Tic complexes, and about different potential regulation routes. In particular the effect of changes in the metabolic redox state on import characteristics was analyzed, since the $NADP^+$/NADPH ratio was already proposed to have an impact on the composition of the Tic complex.

Results

This section is comprised of three chapters, each of which representing an independent study that is either published in or submitted to an international peer-reviewed journal. The main results and conclusions of the manuscripts are summarized below. Furthermore, the contribution of the author to the individual chapters is explained.

Chapter 1: Tic62 – a protein family from metabolism to protein translocation. (2007) by Balsera M, **Stengel A**, Soll J and Bölter, B. *BMC Evol Biol* 7, 43.

In this study, an evolutionary investigation of the Tic62-related protein family was performed, using all available Tic62 homologues. Furthermore, to gain insight into the function of the Tic62-related proteins, a possible 3D-structure of the N-terminal part of Tic62 was analyzed by computational modelling. The results suggest that Tic62 consists of two evolutionary separate modules: the N-terminus is not only found in flowering and non-vascular plants, but also in cyanobacteria and even in green sulphur bacteria and shows a high degree of conservation among all oxyphototrophs. Thus it was proposed to be of ancient origin. In contrast, the C-terminal region, which interacts with FNR exists exclusively in flowering plants. It displays no sequence similarity to any other known motifs and was probably acquired quite recently in evolution. Hence, only flowering plants contain full-length Tic62, all other organisms analyzed possess phylogenetic older, truncated Tic62-like proteins. We suggested that the presence of the FNR-binding domain in flowering plants might be essential for the function of the protein as a Tic component. Furthermore, phylogenetic analyses revealed six Tic62-related protein subfamilies containing sequences from land plants, red and green algae, cyanobacteria and green sulphur bacteria. Additionally, a structurally conserved Rossmann fold, responsible for binding of pyridine nucleotides, was found in the N-terminal module of Tic62, and key residues involved in nucleotide binding and attachment of the protein to the inner envelope membrane of chloroplasts or to the Tic complex were proposed.

I contributed to this study by performing experiments in *Physcomitrella patens* that led to the analysis if Tic62 in non-vascular plants. M. Balsera carried out the acquisition, analysis and interpretation of the *in silico* data. B. Bölter and I participated in the sequence analyses. The manuscript was written by M. Balsera and revised by me, J. Soll and B. Bölter. J. Soll and B. Bölter directed the analyses and content of the manuscript.

Chapter 2: Tic62: Redox-regulated translocon composition and dynamics. (2008) by **Stengel A**, Benz P, Balsera M, Soll J and Bölter B. *J Biol Chem.* 283, 6656-6667.

In this study, the structure, function and redox-related properties of Tic62 were investigated to analyze how the chloroplast redox state influences translocon behaviour and composition. The results clearly show that Tic62 shuttles reversibly between the chloroplast membranes and the stroma, depending on the metabolic $NADP^+$/NADPH ratio. Moreover, this ratio influences the interactions of Tic62 with the Tic translocon and with FNR. Oxidized conditions lead to a stronger attachment of Tic62 to the membrane as well as to the Tic complex. A central region of approximately 100 amino acids was demonstrated to be sufficient for mediating this binding. Reduced conditions on the other hand caused a transition to a soluble state in the stroma and a preferred association with FNR as interaction partner. Additionally it was shown that the N-terminus containing the predicted NADPH binding site is an active *bona fid*e dehydrogenase *in vitro*. To gain information about structural properties of Tic62, circular dichroism measurements were performed which showed that the N- and C-terminus of Tic62 are not only functionally, but also structurally different: the N-terminus displays a mainly α-helical structure that roughly confirmed the computational model from the previous study. In contrast to this, the C-terminus was found to contain mostly disordered features, which suggested that binding to FNR is necessary to gain an ordered three-dimensional structure in this part of the protein. It was proposed that Tic62 possesses redox-dependent properties and is subject to redox regulation itself that would allow it to fulfil the predicted role as redox sensor protein in the chloroplast.

The biochemical experiments describing the changes in the distribution and interactions of Tic62 were performed by me, as well as the sequence analysis and dehydrogenase assays. The binding assays and sucrose density gradient experiments were carried out by me and P. Benz, the import experiments were performed by me and B. Bölter. M. Balsera, P. Benz and I contributed to the spectroscopic CD analyses. The manuscript was written by P. Benz and me and revised by J. Soll, B. Bölter and M. Balsera. J. Soll and B. Bölter directed the study and content of the manuscript.

Chapter 3: Preprotein import into chloroplasts via the Toc and Tic complexes is regulated by redox signals in *Pisum sativum*. (2009) by **Stengel A**, Benz P, Buchanan B, Soll J and Bölter B. *Molecular Plant,* in press

The previous results indicated that the composition of the Tic complex is influenced by redox signals such as the $NADP^+$/NADPH ratio. However, only little was known about any effects of redox signalling on protein import efficiency. To investigate this, import experiments under several redox conditions were performed to analyze a potential redox-based import regulation. The results suggest that protein translocation can be altered by various redox signals: reduction of thiol groups in the Toc receptors and Toc import channel led to a clear import stimulation of proteins that use the general import pathway. This regulation is therefore influenced by the formation/disruption of disulfide bridges. Moreover, changes in the metabolic redox state (*i.e.* the $NADP^+$/NADPH ratio that was already shown to have an impact on the composition of the Tic complex) indeed had an influence on protein import: oxidized conditions (a high $NADP^+$/NADPH ratio) caused an increase in import efficiency. This signal is likely sensed and mediated by the redox-active components of the Tic complex. Interestingly, the data indicated that the import of only a subgroup of precursor proteins is regulated by the stromal redox state, which suggests the existence of several Tic subcomplexes and/or a highly dynamic Tic translocon composition. Furthermore, import experiments into chloroplasts of the non-vascular plant *Physcomitrella patens* and the green algae *Chlamydomonas reinhardtii* allowed us to hypothesize that import regulation via disulfide bridges in the Toc complex is an evolutionary old regulation pathway, in contrast to import regulation by the stromal $NADP^+$/NADPH ratio that was probably acquired more recently in evolution.

I performed all the import experiments, including analyses and quantification of the data, sequence analyses, $NADP^+$ measurements and diagonal SDS redox gels. P. Benz and B. Bölter carried out the cloning of the utilized preproteins. The manuscript was written by me and revised by P. Benz, B. Buchanan, J. Soll and B. Bölter. The study was directed by B. Buchanan, J. Soll and B. Bölter.

Chapter 1

Tic62 – a protein family from metabolism to protein translocation. (2007) by Balsera M, **Stengel A**, Soll J and Bölter, B. *BMC Evol Biol* 7, 43-54.

BMC Evolutionary Biology

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Tic62: a protein family from metabolism to protein translocation

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Abstract

Background: The function and structure of protein translocons at the outer and inner envelope membrane of chloroplasts (Toc and Tic complexes, respectively) are a subject of intensive research. One of the proteins that have been ascribed to the Tic complex is Tic62. This protein was proposed as a redox sensor protein and may possibly act as a regulator during the translocation process. Tic62 is a bimodular protein that comprises an N-terminal module, responsible for binding to pyridine nucleotides, and a C-terminal module which serves as a docking site for ferredoxin-NAD(P)-oxido-reductase (FNR). This work focuses on evolutionary analysis of the Tic62-NAD(P)-related protein family, derived from the comparison of all available sequences, and discusses the structure of Tic62.

Results: Whereas the N-terminal module of Tic62 is highly conserved among all oxyphototrophs, the C-terminal region (FNR-binding module) is only found in vascular plants. Phylogenetic analyses classify four Tic62-NAD(P)-related protein subfamilies in land plants, closely related to members from cyanobacteria and green sulphur bacteria. Although most of the Tic62-NAD(P)-related eukaryotic proteins are localized in the chloroplast, one subgroup consists of proteins without a predicted transit peptide. The N-terminal module of Tic62 contains the structurally conserved Rossman fold and probably belongs to the extended family of short-chain dehydrogenases-reductases. Key residues involved in NADP-binding and residues that may attach the protein to the inner envelope membrane of chloroplasts or to the Tic complex are proposed.

Conclusion: The Tic62-NAD(P)-related proteins are of ancient origin since they are not only found in cyanobacteria but also in green sulphur bacteria. The FNR-binding module at the C-terminal region of the Tic62 proteins is probably a recent acquisition in vascular plants, with no sequence similarity to any other known motifs. The presence of the FNR-binding domain in vascular plants might be essential for the function of the protein as a Tic component and/or for its regulation.

Background

Chloroplasts, together with mitochondria, are the major energy producers in all eukaryotic photosynthetic organisms. The endosymbiotic theory proposes a prokaryotic origin for plastids and mitochondria. During the endosymbiotic process a host cell engulfed distinct ancestral bacteria. Part of the genomes of these endosymbiotic bacteria have been kept and, as a result, plastids and mito-

chondria are the only organelles in the cell containing their own genome. However, while the chloroplast genome is composed of about 120 genes, its proteome is estimated to consist of about 3000 proteins [1]. The development of highly specific organellar transport mechanisms was thus the response to the necessity for re-importing the gene products and to guarantee an optimal communication between cells and organelles.

The general import pathway in chloroplasts involves the cooperation of two heteroligomeric complexes in the outer and inner envelope of chloroplasts, namely the Toc complex–composed of Toc159, Toc75, Toc64, Toc34 and Toc12 subunits–and the Tic complex–made up of Tic110, Tic62, Tic55, Tic40, Tic32, Tic22 and Tic20 subunits–respectively [2]. For a proper import activity, several chaperones in the cytosol (Hsp90 and cHsp70), intermembrane space (isHsp70) and stroma (ClpC and Cpn60) are functionally coordinated during different stages of the transport process [3-5].

Unlike the different protein transport systems in thylakoids, the protein import machinery in the outer/inner envelope membrane of chloroplasts does not show obvious homology to any bacterial secretion system [6]. This is hardly surprising since the bacterial systems were required for thylakoids and therefore a new transport machinery had to be developed in the host cell to maintain the specificity for chloroplast communication. However, sequence analyses indicated that certain components of the translocons in chloroplasts are of bacterial origin. Besides, there is a parallelism in the chaperone system required in some transport stages [6]. The translocation channel in the outer envelope membrane, Toc75, is related to outer membrane proteins involved in the transport or integration of proteins in Gram-negative bacteria [7,8]. Tic20, which is discussed to constitute part of the protein-conducting channel, shares sequence similarities to bacterial amino acid transporters [9]. Other subunits might have been recruited and adapted as they show homology to bacterial proteins not related to transport processes. Tic22, which is thought to mediate the interaction of the Toc and Tic complexes during import, has cyanobacterial counterparts with unknown function and is proposed to be localized in the thylakoid lumen [10]. Some cyanobacterial proteins contain cofactor-binding motifs similar to those found in Tic62, Tic55 and Tic32. Tic55 contains a Rieske iron-sulphur centre and a mononuclear iron-binding site [11], and Tic62 and Tic32 each have a NAD(P)-binding motif [12,13]. No prokaryotic counterparts have been detected by direct sequence comparison for the other subunits that compose the translocons, which may indicate that they have evolved from the proteome of the ancestral host to fulfil specific functions demanded after the development of plastids and to ensure the specificity of the transport process in the outer/inner envelope membranes of chloroplasts.

Genome-wide analyses had shown that some subunits of the translocons (Toc75, Toc159, Toc34, Tic20) are encoded by more than one gene in *Arabidopsis thaliana* [14,15]. Experimental data derived from analyses of the isoforms of the Toc complex revealed that the different members associate with structurally and developmentally distinct import complexes. Four homologues compose the Toc75 family in *Arabidopsis thaliana* (atToc75-I, atToc75-III, atToc75-IV and atToc75-V). The gene encoding the functional orthologue of Toc75 from *Pisum sativum*, atToc75-III, is essential for the viability of plants from the embryonic stage. This is not the case for atToc75-IV, which could play a role during growth in the dark. It seems that atToc75-I is in fact a pseudogene [16]. The function and relation with the Toc machinery of atToc75-V is still a matter of intensive study. In the case of Toc34 and Toc159 families, two (atToc33 and atToc34) and four (atToc159, atToc132, atToc120 and atToc90) isoforms are identified in the *Arabidopsis* genome, respectively. Whereas atToc33 associates preferably with atToc159, atToc34 does with atToc132/atToc120 and this association is likely related to the import of photosynthetic and non-photosynthetic precursors, respectively [17,18]. Four homologues are identified for Tic20 in *Arabidopsis* and only two of them contain a predicted transit peptide. However, the function and subcellular localization of the two Tic20 homologues with non-predicted transit peptide are still unknown [14].

In spite of the wealth of information about the Toc complex, less is known about phylogenetic relationships of the Tic complex subunits. Here we take a closer look at the structure, function and evolution of one component of the Tic complex, Tic62. The N-terminal module of Tic62 has a conserved NAD(P)-binding site and its C-terminal region was found to interact with ferredoxin-NAD(P)-oxido-reductase (FNR) [12]. Homology searches and phylogenetic analyses show that the N-terminal domain is highly conserved among all oxyphototrophs and green sulphur bacteria. However, the C-terminal region (FNR-binding domain) is only found in vascular plants. Phylogenetic analyses indicate that there are four groups of Tic62-NAD(P)-related proteins in land plants. The first group is orthologous to the reported Tic62 from pea [12]. The physiological roles of the Tic62-NAD(P)-related proteins in the cell remain to be shown.

Results and discussion

Tic62, a protein of 62 kDa that is part of the Tic complex, has been proposed to function as a sensor protein whose possible role is to regulate protein import into chloroplasts by sensing and reacting to the redox state of the

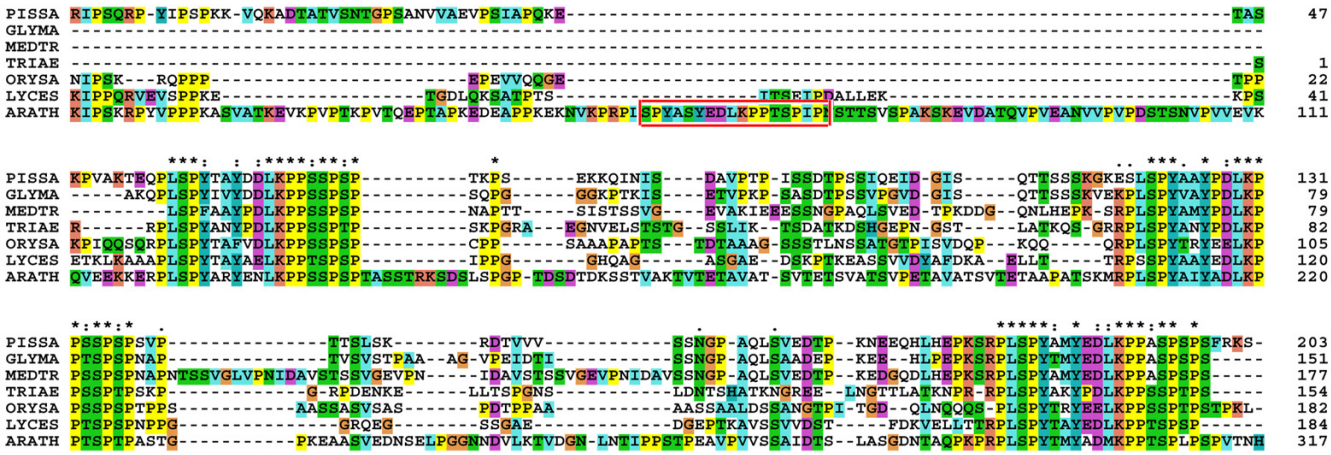

Figure 1

Multiple sequence alignment of the C-terminal domain of the Tic62 protein family from vascular plants. The multiple sequence alignment shows the FNR-binding domain in vascular plants (residues 387–534 in psTic62). Three repetitive motifs, S-P-Y-x (2)-Y-x-D/E-L-K-P (2)-S/T/A-S/T-P-S/T-P, involved in the binding of FNR [12] are highly conserved within the family. A fourth repetition found just in *Arabidopsis* sequence is marked in a box. The pea (PISSA), *Arabidopsis thaliana* (ARATH) and *Oryza sativa* (ORYSA) sequences were retrieved from GenBank. The sequences from tomato (LYCES), *Glycine max* (GLYMA), *Medicago truncatula* (MEDTR) and *Triticum aestivum* (TRIAE) were identified in dbEST and retrieved from plantGDB. The representation of the alignment is the standard from the ClustalX program [43].

organelle. So far the only Tic62 protein studied is that from *Pisum sativum* [12]. This protein was found to have two functional modules: the N-terminus was shown to bind pyridine nucleotides and the C-terminal region interacts with FNR. The FNR-binding module consists of a repetitive, highly conserved KPPSSP motif. One or two transmembrane helices were proposed for the pea sequence and both the N- and the C-terminus seem to face the stroma [12].

Excluding the transit peptide, psTic62 consists of 470 residues. The blast search against the protein databases with psTic62 [Swiss-Prot:Q8SKU2] as a template resulted in several sequences from which just two correspond to the full-length form of the mature psTic62: one from *Arabidopsis thaliana* [GenBank:NP_188519] and another from *Oryza sativa* [GenBank:ABG65881]. All the other hits, which showed recognizable sequence similarity to the N-terminal NAD(P)-binding domain of Tic62, lack the C-terminal module (residues 387–534) responsible for the FNR binding and represent a short version of the Tic62 protein. A search of the FNR-binding motif over dbEST revealed its presence exclusively in vascular plant organisms (*e.g.*, *Lycopersicon esculentum*, *Medicago truncatula*, *Triticum aestivum*, *Glycine max*, *Lotus japonica*) (Figure 1).

Interestingly, all the proteins homologous to the NAD(P)-binding part of Tic62 were from photosynthetic organisms (green plants, oxyphotobacteria, and green sulphur bacteria). A multiple sequence alignment of these proteins is shown in Figure 2. A phylogenetic tree was built based on the alignment (Figure 3). Both the multiple alignment and the phylogenetic tree indicate that the Tic62-NAD(P)-related protein family is made up of four well-supported clusters (support values of 100/95, 100/68, 100/100 and 100/100, Figure 3) that have been divided into six groups. These groups are schematically represented in Figure 4 and are described below. The four plant subfamilies are classified according to the GenBank accession number of the *Arabidopsis* protein found within each group (the locus_tag of the *Arabidopsis* gene is shown in parenthesis).

(i) Group I: NP_188519 (At3g18890). This subfamily contains the original Tic62 sequence from pea and makes up the Tic62 family, even though not all the members of this family have a molecular weight of 62 kDa and the association with the Tic machinery remains to be shown (see below). It is composed of proteins from chloroplast-containing organisms of land plants and red algae. So far no sequence of this subfamily was found in green algae (Ostreococcus or Chlamydomonas), in the diatom Thalassiosira or in any oxyphotobacteria. Because a final annotation of the green algae genomes is still in progress, a final confirmation of the absence of the protein of group I in green algae is pending. This group is characterized by the motif E-R-P/A-T-D-X-Ar-K/G-E-T-H (residues 350–371 in Figure 2), where Ar represents an aromatic residue. Surprisingly, only the sequences from vascular plants within this group show the full-length version of the Tic62

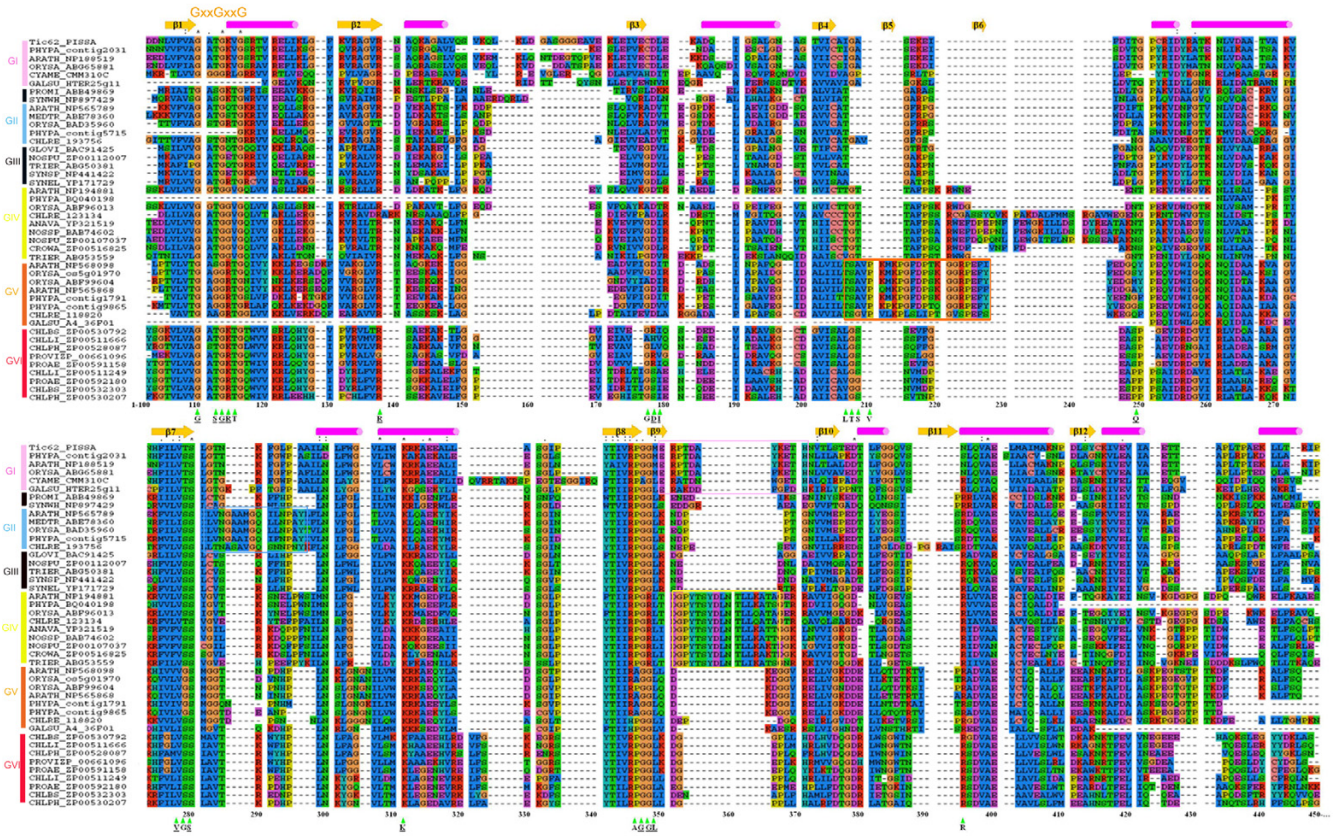

Figure 2

Multiple sequence alignment of the N-terminal domain of the Tic62-NAD(P)-related protein family. A multiple sequence alignment of the N-terminal domain (residues 87–334 in psTic62) of representative members of each of the six groups of Tic62-NAD(P)-related sequences was performed with ClustalX. Above the alignment the site of the GxxGxxG motif and the known secondary structure of the NP_568098 sequence from *Arabidopsis* [PDB:1XQ6] are displayed. α-helices and β-strands are represented by cylinders and arrows, respectively. The positions of the residues involved in the binding of NADP are marked with a triangle, and the identities of the residues from the crystal structure are indicated. The conserved residues between 1XQ6 and psTic62 are underlined. The sequence motifs that distinguish each group are shown in a box (see Results and Discussion). In the alignment the sequences are indicated with an abbreviation of the name of the organism followed by its identifying access code in the databases: ANAVA, *A. variabilis* ATCC 29413; ARATH, *A. thaliana*; CHLBS, *C. phaeobacteroides* BS1; CHLLI, *C. limicola* DSM 245; CHLPH, *C. phaeobacteroides* DSM 266; CHLRE, *C. reinhardtii*; CROWA, *C. watsonii* WH 8501; CYAME, *C. merolae*; GALSU, *G. sulphuraria*; GLOVI, *G. violaceus* PCC 7421; MEDTR, *M. truncatula*; NOSPU, *N. punctiforme* PCC 73102; NOSSP, *Nostoc sp.* PCC 7120; ORYSA, *O. sativa*; PHYPA, *P. patens*; PROAE, *P. aestuarii* DSM 271; PROMI, *P. marinus* str. MIT 9312; PROVI, *P. vibrioformis* DSM 265; SYNEL, *S. elongatus* PCC 6301; SYNSP, *Synechocystis sp.* PCC 6803; SYNWH, *Synechococcus sp*. WH 8102; TRIER, *T. erythraeum* IMS101. Note that the sequences from GALSU (GI and GIV), PHYPA (GII and GIII) and CHLRE (GIII and GIV) are incomplete sequences and lack part of the N- and C-terminal region. The representation of the alignment is the standard from the ClustalX program [43].

protein and contain the FNR-binding motif at the C-terminus. A minor distinction between Tic62 from Arabidopsis and the other full-length sequences is the number of four or three repetitive modules, respectively (Figure 1). Exhaustive searches for the FNR-interacting repeat in the Physcomitrella patens genome revealed no hits to these regions. 3'RACE PCRs of the detected Physcomitrella Tic62 gene were performed to determine its C-terminal sequence. It resulted exclusively in the short form of the gene, giving a stop codon in position 259. Additionally, immunodecoration with the pea Tic62 antibody, raised against the C-terminal part of the protein (residues 412–534), showed no signal in Physcomitrella chloroplasts (data not shown). Finally, an insertion of 6–15 residues (positions 148–168 in the alignment, Figure 2) is found in vascular plants and red algae. The search of this motif in the Physcomitrella genome resulted in no hits. The overall identity of the sequences composing this subfamily is 40%. All of them contain a transit peptide for targeting the protein to chloroplasts, and they might be

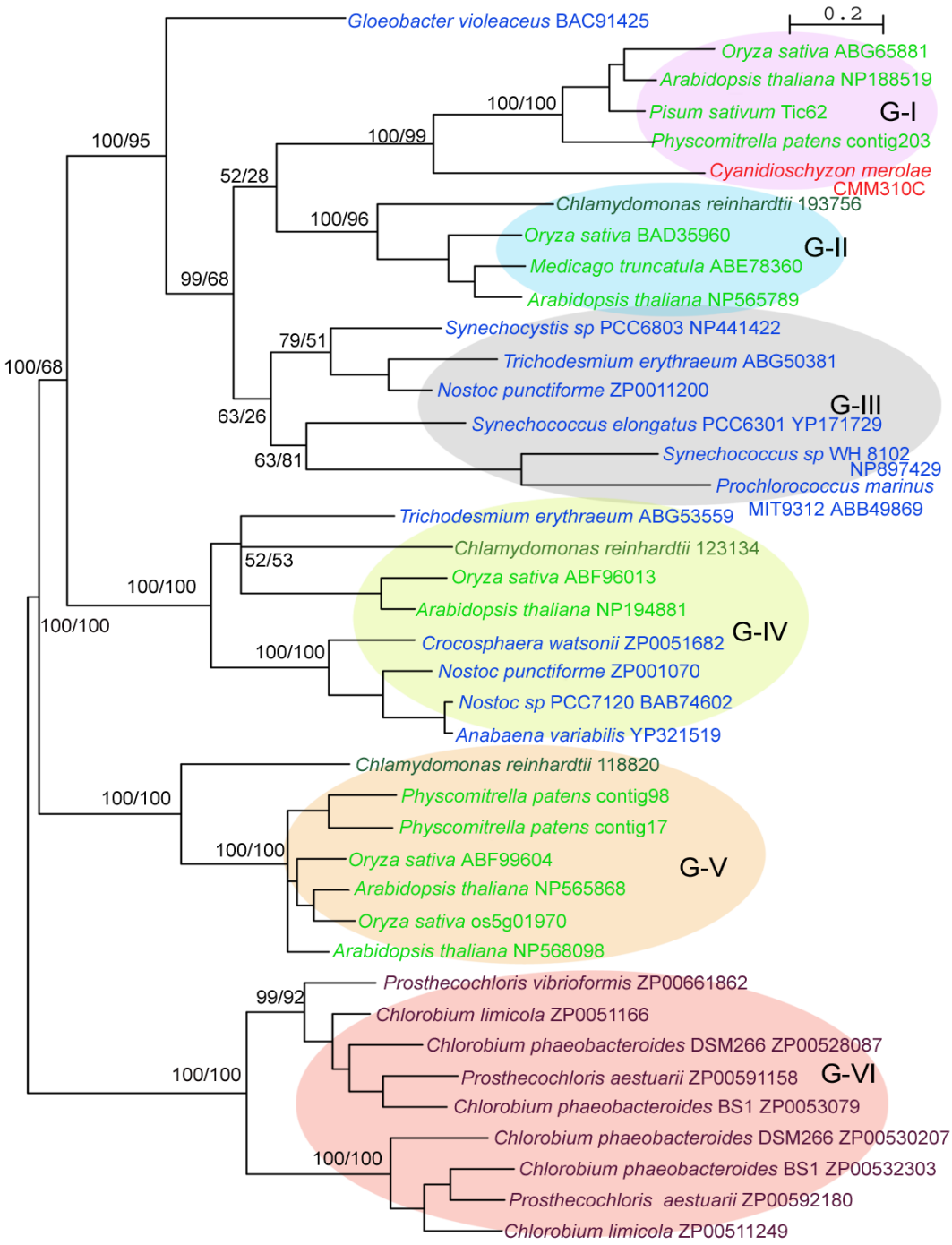

Figure 3

Phylogram of representative members of the Tic62-NAD(P)-related family. The optimal unrooted phylogenetic tree obtained by MrBayes is shown for representative members of the Tic62-NAD(P)-related family. The topology predicted with Bayesian and ML methods were not different from each other and four well-supported clusters and six groups are recognized. For display purposes, the green sulphur bacteria have been used as outgroup. The Bayesian posterior probability percentage (pP%) and the bootstrap values obtained by PhyML are shown in the nodes (Bayesian/ML). The organism's name is indicated followed by the accession number of the protein in the databases. Land plants, green algae, red algae, cyanobacteria and green-sulphur bacteria are coloured in light green, dark green, red, blue and brown, respectively. Branch lengths are proportional to evolutionary distances.

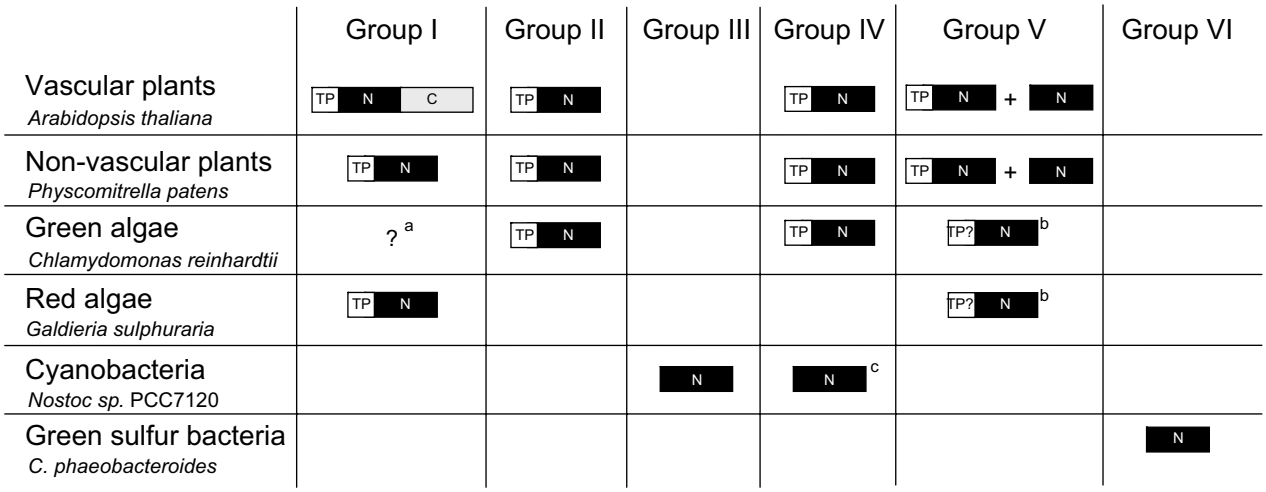

Figure 4

Presence of Tic62-NAD(P)-related proteins in cyanobacteria, algae, land plants and green sulphur bacteria. Tic62 is a bimodular protein (Nt and Ct modules for NAD(P) and FNR binding, respectively) with a transient transit peptide (TP) for importing into chloroplasts. The presence of the modules and/or the transit peptide is indicated for the Tic62-NAD(P)-related proteins. A question mark indicates that the complete genome is not available and: [a] the absence of the protein cannot be assured; [b] the existence of a transit peptide is not known. [c]Proteins corresponding to group IV are only found in unicellular and filamentous diazotrophic cyanobacteria and not in others such as *Gloeobacter violaceus*, *Prochlorococcus marinus* or *Synechocystis sp.* PCC 6803 (see text). The accession numbers in the corresponding database (indicated in brackets) for the proteins found in this figure are the following: *Arabidopsis thaliana* (GenBank), NP_188519 (group I), NP_565789 (group II), NP_568098/NP_565868 (group IV, without/with transit peptide), NP_194881 (group V); *Physcomitrella patens* (PhyscoDB), contig 2031 (group I), contig 5715 (group II), contig1791/contig9865 (group IV, without/with transit peptide), BQ040198 (group V); *Chlamydomonas reinhardtii* (ChlamyDB), 193756 (group II), 118820 (group IV), 123134 (group V); *Galdieria sulphuraria* (The *Galdieria sulphuraria* Genome Project), hter25g11 (group I), A436F01 (group IV); *Nostoc sp.* PCC7120 (GenBank), ZP_00112007 (group III), BAB74602 (group IV); *Chlorobium phaeobacteroides* (GenBank), ZP_00528087 (group VI).

localized at the inner envelope membrane as it was previously reported for the pea sequence [12], though the FNR-binding domain could modulate the subcellular localization of the protein by the interaction with FNR and/or other proteins.

(ii) Group II: NP_565789 (At2g34460). The members of this subfamily are homologous to the short version of the Tic62 protein from vascular plants. This subfamily is composed of proteins from the green algae *Chlamydomonas reinhardtii*, and both non-vascular and vascular plants (*Physcomitrella* and *Arabidopsis*, respectively). Surprisingly, there is no sign of the presence of members of this group in red algae genomes (*Galdieria*, *Porphyra* or *Cyanidioschyzon*). This group is closely related to group I and the phylogenetic tree is highly consistent in splitting up these two groups (Figure 3). The green plant sequences of this group are characterized by the motif L-V-N-G-A-A-p-G-Q-x(2)-N-P-A-Y, where p represents a polar residue (range 282–296 in Figure 2). The proteins from green plants contain an N-terminal extension, which is predicted to act as a transit peptide to target the proteins to chloroplasts. Recently, the *Arabidopsis* protein has been identified in a proteomic analysis of isolated plastoglobules [19].

(iii) Group III. This cyanobacterial subfamily is composed of proteins from a variety of organisms such as *Synechocystis* sp. PCC 6803, the small-genome cyanobacteria *Prochlorococcus marinus* (MIT9313, SS120 and MED4) or the heterocystous cyanobacteria *Nostoc* sp. This group comes together with groups I and II in a well-supported cluster (support values 100/95) and the phylogenetic trees were highly consistent in outgrouping the sequence from *Gloeobacter violaceus* (Figure 3), a cyanobacterial member of an early branching lineage [20]. Due to the annotation in the databases of the *Synechocystis* sequence from this family (NP_441422, sll1218) as ycf39 gene product, a connection between Tic62 and ycf39 was previously proposed [12]. However, it can be traced that the original ycf39 gene product is not related to sll1218 but to slr0399 in *Synechocystis* [GenBank:NP_441851] [12]. Both cyanobacterial proteins share 26% identity and 42% similarity.

The ycf39 gene product (slr0399) was found to act as a chaperone for quinone binding [21]. This cyanobacterial protein is similar to the NP_195251*Arabidopsis* sequence that is not a Tic62-NAD(P)-related protein. Therefore, it can be argued that a connection between Tic62 and ycf39 may be an artefact originated by a non-reliable annotation in the protein database.

(iv) Group IV, NP_194881 (At4g31530). This group is made up of proteins from green plants and, interestingly, only unicellular and filamentous diazotrophic cyanobacteria. The members of this family also represent short versions of the Tic62 protein. So far no sequences from red algae were found. This group is characterized by the motif G-P-Y-T-S-Y-D-L-N-T-L-L-K/Q-A-T/K-A/S/T (range 353–377 in Figure 2). The land plant sequences contain a predicted chloroplast transit peptide and proteomics studies have localized the protein from *Arabidopsis* in chloroplasts [22]. The lack of homologous sequences in other cyanobacteria such as *Synechocystis* or *Prochlorococcus* may be in accordance with a previously reported work, which showed that *Nostoc* proteins have higher similarity to *Arabidopsis* nuclear-encoded proteins than proteins from *Prochlorococcus* or *Synechocystis* [23].

(v) Group V, NP_568098 (At5g02240). This family consists of proteins exclusively from eukaryotic phototrophs, which show homology to the short version of Tic62. The sequences of the land plant members of this group are characterized by the motif T-S-A-V-P-K-M-K-P-G-F-D-P-S/T-K-G-G-R-P-E-F-h, where h represents a hydrophobic residue (range 206–227 in Figure 2). Two different subgroups of sequences within this subfamily in land plants were identified, which are differentiated by the presence of a predicted transit peptide. This may suggest a dual localization in the cell for the members of this group. The first subgroup comprises land plant proteins without a predicted transit peptide. These proteins are [GenBank:NP_568098] (At5g02240), [GenBank:AAK73149] (Os03g60740) and [PhyscoDB:contig1791] from *Arabidopsis*, rice and *Physcomitrella*, respectively. The second subgroup is composed of sequences that contain a predicted transit peptide for chloroplasts: [GenBank:NP_565868] (At2g37660), [GenBank:ABF99604] (Os05g01970) and [PhyscoDB:contig9865] from *Arabidopsis*, rice and *Physcomitrella*, respectively. At2g37660 has been found in chloroplasts by proteomics analyses [22]. In spite of a possible difference in localization, the two subgroups are highly similar (*e.g.*, 79% identity between NP_568098 and NP_565868 in *Arabidopsis*) which suggests a similar function in the cell. Only incomplete sequences were found in the algae genomes analysed (*Chlamydomonas* and *Galdieria*) and, therefore, no further conclusions can be made for these organisms.

The structure of the NP_568098*Arabidopsis* protein bound to NADP has been recently solved at 1.8 Å resolution by X-ray crystallography [PDB:1XQ6]. The residues involved in the binding to the cofactor are marked with a triangle in the multiple sequence alignment (Figure 2).

(vi) Group VI. The last group to be mentioned corresponds to proteins of green sulphur bacteria (Figure 2 and Figure 4). Two subgroups are recognized, which likely originated from a gene duplication event (Figure 3). The similarity search using psTic62 as a template retrieved sequences from green sulphur bacteria with homology to the short version of Tic62. These are anoxygenic phototrophic bacteria that contain a type-I (Fe-S) reaction centre. A reverse blast search, using the green sulphur Tic62-related sequences, did not retrieve any sequences from oxyphotosynthetic organisms different from the groups mentioned above. Although very different organisms, the genome comparison between green sulphur bacteria and oxyphotosynthetic organisms showed that many components of photosynthesis and energy metabolism are highly similar. Green sulphur bacteria, cyanobacteria and eukaryotic phototrophs are the only organisms that synthesize chlorophyll *a* and also directly reduce pyridine nucleotides[24].

The presence of so many proteins in chloroplasts related to the NAD(P)-binding domain of Tic62 deserves a detailed study. The function of the N-terminal module seems important for the viability of the photosynthetic organisms since the gene has been conserved in all the genomes. All the proteins are predicted to bind pyridine nucleotides and are referred here as Tic62-NAD(P)-related family due to the similarity to the NAD(P)-binding domain of psTic62. The Tic62-NAD(P)-related family is of ancient origin, as proteins were not only found in ancient cyanobacteria (*Gloeobacter violaceus*) but also in green sulphur bacteria. This might propose that a Tic62-NAD(P)-related protein was already present in the ancestor who evolved to green sulphur bacteria and cyanobacteria. The presence of two genes in *Nostoc punctiforme* (groups III and IV) might suggest that a gene duplication event occurred prior to the evolution of cyanobacteria (Figure 3). Some cyanobacterial organisms could have lost one of the genes, which could explain its absence in *Gloeobacter*, *Prochlorococcus* and *Synechocystis* in group IV. Two highly supported groups (I and II) together with group III comprise a big cluster of sequences and groups I and II are possibly derived from group III which contains the majority of the cyanobacterial proteins. A four-cluster likelihood-mapping analysis (cluster a = group I+II, cluster b = group III, cluster c = group IV, cluster d = group V or cluster a = group I, cluster b = group II, cluster c = group IV, cluster d = group V) showed that branching order (a, b)–(c, d) was favoured in more than 90% of 10,000 puzzling, and dem-

onstrated that group V is closely related to group IV. The presence of paralogues in land plants of group V could be due to a gene duplication event within the eukaryotic organism.

Most of the Tic62-NAD(P)-related proteins in higher plants are found in chloroplasts, but only the specific localization of psTic62 (group I) at the inner envelope membrane of chloroplasts and NP_565789 (At2g24460, group II) in plastoglobules have been shown experimentally [12,19]. It would be worth investigating the subcellular localization of the other members of the family and, especially, to analyse the possible dual localization of the proteins belonging to group V. The lack of a transit peptide had also been described in two homologues that compose the Tic20 family in *Arabidopsis* [14]. A possible localization outside plastids could be another example of a protein of cyanobacterial origin that has been redirected to a compartment different from plastids [23]. However, the targeting information to chloroplasts could be different from the canonical transit peptide [22,25,26] and a localization of such proteins in chloroplasts cannot be excluded. The presence of members of the family at the inner envelope membrane of chloroplasts, involved in the import process, and in plastoglobules, structures that act as a functional metabolic link between the inner envelope and thylakoid membranes, points to an important role of the protein family in metabolism.

The resolution of the structure of a protein is a major step in understanding the function. Since the similarity among sequences in the Tic62-NAD(P)-related family is sufficiently high, the knowledge of the structure of one member of Tic62-related family permits to draw general conclusions about the structure of other members. The crystallized NP_568098 protein shows the typical NADPH-Rossman fold. Figure 2 also represents the secondary structure of the crystallized NP_568098 protein. Clearly, most of the insertions and deletions of the proteins in this family correspond to loops in the crystal structure and most of the motifs related to α- and β-conformations are highly conserved. Therefore, the NADPH-Rossman fold is also expected for the core structure of all members of the Tic62-NAD(P)-related family, with differences mainly in the loop regions. The glycine-motif in the coenzyme-binding region is fully conserved in the whole family (GxxGxxG, range 111–116 in Figure 2) and it may be related to the extended short-chain dehydrogenase-reductase superfamily [27]. The highly conserved aspartic acid residue required for stabilization of the adenine-binding pocket is found in the loop between β3 and α3, except for group VI [28]. However, large differences are expected in the regions of the β5 and β6 strands. In the crystal structure, these two β-strands form an antiparallel β-sheet, which connect a long loop (Figure 2; see below). The differences in this region among the subfamilies could be correlated to the specific function of each subfamily. Since the protein was crystallized in the presence of NADP, the residues involved in the binding to the cofactor were identified (G11, S13, G14, R15, T16, R38, G55, D56, I57, L76, T77, S78, A79, V80, Q103, V131, G132, S133, K155, A174, G175, G176, L177, R205; for underlined residues see below). These residues are marked in Figure 2. From the multiple sequence alignment it can be concluded that many residues that bind to NADP are highly conserved within the family (9 out of 22). Specifically, the conservation of the residues (or their physicochemical properties) involved in the NADP binding is high in members of group I (14 out of 22). These residues are underlined above and they could represent the residues implicated in the NADP binding of Tic62. Mutagenesis studies are necessary to establish the role of these residues clearly.

The mode of interaction of Tic62 with the membrane/Tic complex is unknown. Previous experiments showed that likely hydrophobic contacts mediate the binding to the membrane/Tic complex, as most of the protein remains within the membrane upon alkaline and urea treatments [12]. TMHMM [29] and PredictProtein [30] algorithms do not predict any transmembrane helices in group I. Moreover, protease digestion experiments showed that psTic62 is protected in inner envelope vesicles that, together with the hydrophilic profile of Tic62, suggest that the protein faces the stroma while attached to the membrane/Tic complex. Based on the identity (27% identity; 41% similarity) between atTic62 (NP_188519) and NP_568098 [PDB:1XQ6], a homology model procedure was followed to construct a model for the NADP-binding domain of the Tic62 protein (residues 78–331 in atTic62; see additional file 1: PDB coordinates for the atTic62 model). Figure 5a shows the sequence alignment of the N-terminal domain of the atTic62 protein and the template based on the multiple sequence alignment of the Tic62-NAD(P)-related family (Figure 2). The key residues involved in the pyridine ring binding are shown in red. The predicted secondary structure of atTic62 is compared with the known secondary structure of the template. As can be seen, most of the conformational elements are conserved in both sequences. Slight differences are the presence of β5 and β6 strands in the template (as mentioned above), and two smalls α-helices predicted between β2 and β3 strands in atTic62. A model was built based on this alignment and it was structurally evaluated with WHATCHECK. The corresponding values were good: Ramachandran plot, -2.215; backbone conformation, -3.761; chi-1/chi-2 rotamer normality, -1.150; bond lengths, 0.716; bond angles, 1.439. Only the values for the backbone conformation were poor, but this is probably due to gaps in the alignment and located in loop regions of the template (Figure 5a). In

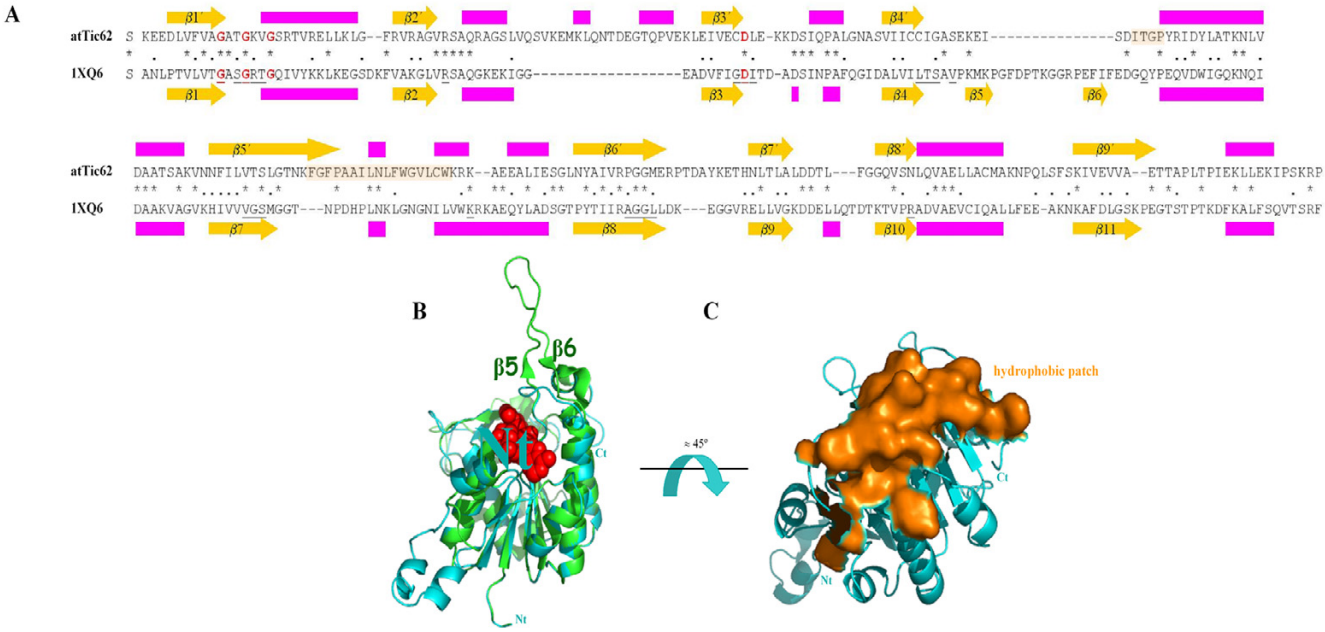

Figure 5
3D structural model of the N-terminal domain of Tic62 from *Arabidopsis thaliana*. The model was built by homology modelling using the 1XQ6 structure as a template. (a) Sequence alignment of the N-terminal domain of the atTic62 (residues 78–331) protein and the template. The predicted and known secondary structure of atTic62 and the template (1XQ6) are shown. Purple represents α-helix and yellow denotes β-strand. Residues involve in NADP-binding are underlined. The conserved aspartic acid residue required for stabilization of the adenine-binding pocket is found at the end of β3. For residues shade in orange see below; (b) Proposed structural model for atTic62 (cyan) superimposed onto the template (green). NADP ligand is shown in red. Slight differences are expected among subfamilies (e.g., absence of β5 and β6 in Tic62); (c) The hydrophobic region in Tic62 that might attach the protein to the membrane/Tic complex (residues 180–184 and 217–233 in atTic62) is shown in orange.

fact, the structural analysis obtained by the VERIFY3D program assigns positive values all over the structure, except in the regions LQNTDEGT and FPAAILNLFWGVLC in atTic62 (minimum value of -0.16) that support the previous proposal. The energetic parameter of the model was E = -4082.780 kJ · mol^{-1}. In Figure 5b, a view of the proposed structural model for at Tic62 (blue) superimposed to the template (green) is despicted. The NADP ligand is shown in red and the residues involved in the binding are underlined in Figure 5a. It can be seen that the β5 and β6 elements connect a long loop that is missing in atTic62 (Figure 5a and 5b). Interestingly, a large number of hydrophobic residues is concentrated in this region in atTic62 (Figure 5c, marked in orange). The model presented here for atTic62 suggests that the hydrophobic region (residues 180–184 and 217–233 in atTic62 sequence in Figure 5c, which correspond to residues 247–251 and 291–310 in the alignment shown in Figure 2) might be responsible for attaching the protein to the inner envelope membrane of chloroplasts or to the Tic complex, and this region would establish differences in the localisation within cells between the two groups of proteins (template and model). By this way, Tic62 would be attached to the membrane, without spanning it, exposing the two functional modules to the stromal side. The hydrophilic profile and the large number of conserved proline residues at the C-terminal domain make it a better candidate for protein-protein interactions rather than for insertion into the membrane [31]. These interactions might also contribute to the binding of Tic62 to the membrane/Tic complex.

Focusing on group I, one of the questions to be answered is whether or not all members of this group are Tic components. Although they might share a common dehydrogenase activity at the N-terminus, the origin of the FNR-binding module at the C-terminus in vascular plants remains unknown and different functions might be expected among the different organisms. No similar sequences to the C-terminus of psTic62 were found in the databases with significant homology, which could indicate that either the FNR-binding module was lost during evolution and only kept in vascular plants, or (more probably) the FNR-binding module was recently acquired by vascular plants. The high similarity of the NADP-binding domain of the *Physcomitrella* sequence in group I with psTic62 (68% identity) suggests that the short version of

Tic62 in *Physcomitrella*, together with Tic110/Tic55/Tic40/Tic32/Tic22/Tic20 [32], might be a constituent of the Tic complex. The same might be true for *Cyanidioschyzon merolae*. On the other hand, it cannot be excluded that the concurrence of both N-t and C-t domains, or even the FNR-binding domain alone, were compulsory to settle a protein as a Tic component. Further studies are necessary to establish the mode of interaction of Tic62 with the Tic complex in vascular plants and to elucidate the localization and function of members of group I in non-vascular plants.

Still the question remains of the presence and function of FNR at the inner envelope membrane of chloroplasts. In chloroplasts, this protein is found either soluble in the stroma in a non-functional state or attached to the thylakoids, where the protein is involved in the last stage of the electron transport process in photosynthesis. FNR is a ubiquitous flavoenzyme whose function is not exclusively confined to photosynthesis [33] and, recently, the protein has also been found to be localized at the inner envelope membrane of chloroplasts [12]. When attached to the thylakoids, a reductase-binding protein (BP) mediates the binding to the membrane [34]. In line with this, one possibility could be that FNR is attached to the inner envelope membrane via the FNR-binding motif of Tic62. This interaction in vascular plants could be affected by the activity of Tic62 that could specifically regulate the–yet unknown–functional state of FNR in the inner envelope membrane of chloroplasts. The opposite effect cannot be excluded, and the binding of FNR could regulate the activity of Tic62, and therefore the transport machinery. This regulation upon binding could depend on the redox state of chloroplasts and might involve NADP(H)/NAD(H) or a low potential electron donor and another substrate not yet identified [33]. On the other hand, a possible electron transfer process between FNR and Tic62 cannot be excluded although the capacity of Tic62 as electron acceptor/donor has not yet been proven. It is likely that the FNR-binding domain is important for some kind of metabolic regulation just in vascular plants, which needs further studies.

Conclusion

The reported results show that the N-terminal module of Tic62 (NAD(P)-binding domain) is highly conserved among all oxyphototrophs. The Tic62-NAD(P)-related sequences are of ancient origin, since the protein was not only found in cyanobacteria but also in green-sulfur bacteria. This protein family would belong to the extended family of short-chain dehydrogenases-reductases and likely contains the structurally conserved Rossman fold. On the other hand, the C-terminal module in Tic62 (FNR-binding domain) is only found in vascular plants. This domain is enriched in proline amino acids and it would be important for protein-protein interactions that might regulate the function of Tic62 protein. Tic62 proteins in vascular plants would be attached to the inner envelope membrane of chloroplasts, without spanning it, exposing both C-terminal and N-terminal domains to the stroma. Further studies are necessary to establish the mode of interaction of Tic62 with the Tic complex in vascular plants and to elucidate the localization and function of related members in non-vascular plants

Methods

A sequence homology search (tblastn/blastp) was performed using the Tic62 protein sequence from pea (psTic62) as a template (e-value < 10^{-9}). The following biological databases were considered: the non-redundant GenBank database (nr) [35]; the public available *Physcomitrella patens* EST database, PhyscoDB [36]; the genomic database containing the so far sequenced *Physcomitrella patens* genome (access due to collaboration with Prof. Ralf Reski, University of Freiburg); the annotated genome of the red alga *Cyanidioschyzon merolae* [37]; the annotated genome of the green alga *Chlamydomonas reinhardtii*, ChlamyDB [38]; the genome database for plants, plantGDB [39]. Also the following databases were considered: the red algae *Porphyra yezoensis* [40] and *Galdieria sulphuraria* [41] databases; the chlorophyta *Ostreococcus lucimarinus* database [42]; the EST GenBank database (dbEST) [35]. All the retrieved sequences were aligned with the ClustalX program [43], visually inspected and manually corrected. The prediction of the subcellular localization of the proteins was performed with TargetP [44], ChloroP [45] and Predotar [46] programmes.

ProtTest v1.3 [47] was used to estimate the best model of amino acid evolution for phylogeny. The WAG+I+Γ model was chosen using either AIC or BIC as statistical frameworks. Phylogenetic trees were generated on the basis of the maximum-likelihood (ML) and Bayesian analysis using PhyML v2.4.4 [48] and MrBayes v3.1.2 [49] programmes. For ML analysis, four Gamma-distributed sites were considered, and the parameters were estimated from the data. Non-parametric bootstrap values were calculated for ML analyses (100 replicates) to assess the significance of the resulting tree. Bayesian analysis was performed under the same model. Four chains were run for one million generations with sampling every 100 generations. Bayesian posterior probabilities were calculated from the majority rule consensus of the tree sampled after the initial burn-in period corresponding to 2,500 generations. Four-cluster likelihood-mapping [50] implemented in Tree-puzzle v5.2 [51] was performed with 10,000 randomly chosen quartets.

A 3D model for all non-hydrogen atoms was obtained for the N-terminal domain of the mature Tic62 from *Arabi-*

dopsis thaliana (atTic62; NP_188519) by homology modelling using the known 3D structure of NP_568098*Arabidopsis* protein [PDB:1XQ6] as a template. The model was built using the SWISS-MODEL automated modelling server [52] and it was evaluated using WHATCHECK [53], PROMODII [54] and VERIFY3D [55]. The secondary structure prediction of atTic62 was performed using PSIPRED server [56].

Authors' contributions

MB carried out the acquisition, analysis and interpretation of the data, and drafted the manuscript. AS performed the RACE-PCR and immunodecoration experiments in *Physcomitrella patens*. AS and BB participated in the sequence analyses and contributed to each draft of the manuscript. JS directed the study and content of the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

PDB coordinates for the atTic62 model. Model of atTic62 built by homology modelling using 1XQ6 structure as a template.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2148-7-43-S1.pdb]

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Chapter 2

Tic62: Redox-regulated translocon composition and dynamics. (2008) by **Stengel A**, Benz P, Balsera M, Soll J and Bölter B. *J Biol Chem.* 283, 6656-6667.

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TIC62 Redox-regulated Translocon Composition and Dynamics*

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The preprotein translocon at the inner envelope of chloroplasts (Tic complex) facilitates the import of nuclear-encoded preproteins into the organelle. Seven distinct subunits have been identified so far. For each of those, specific functions have been proposed based on structural prediction or experimental evidence. Three of those subunits possess modules that could act as redox-active regulatory components in the import process. To date, however, the mode of redox regulation of the import process remains enigmatic. To investigate how the chloroplast redox state influences translocon behavior and composition, we studied the Tic component and the putative redox sensor Tic62 in more detail. The experimental results provide evidence that Tic62 can act as a *bona fide* dehydrogenase *in vitro*, and that it changes its localization in the chloroplast dependent on the NADP$^+$/NADPH ratio in the stroma. Moreover, the redox state influences the interactions of Tic62 with the translocon and the flavoenzyme ferredoxin-NADP$^+$ oxidoreductase. Additionally, we give initial experimental insights into the Tic62 structure using circular dichroism measurements and demonstrate that the protein consists of two structurally different domains. Our results indicate that Tic62 possesses redox-dependent properties that would allow it to fulfill a role as redox sensor protein in the chloroplast.

Redox regulation plays a crucial role in virtually all metabolic or developmental pathways present in the chloroplast. Numerous studies have presented evidence that electron transfer processes are not only restricted to the photochemical reactions linked to photosynthesis, providing the reducing power for *e.g.* carbon fixation, NO_2^- assimilation or fatty acid biosynthesis, but are also prominent in a regulatory manner. They activate or inactivate many metabolic enzymes (*e.g.* via the thioredoxin/glutaredoxin systems, Ref. 1) or act as part of a signaling pathway to the nucleus to regulate transcription (2). Furthermore, the involvement of thioredoxins, *e.g.* in the regulation of chloroplast translation and protein folding, has been demonstrated (3), and thus redox signaling and regulation seem to influence many steps from transcription to post-translational enzyme activity.

An indication that protein import to the chloroplasts is also regulated by a redox-related process in a substrate-dependent manner was demonstrated by Hirohashi *et al.* (4), who reported a differential import behavior of the non-photosynthetic ferredoxin (Fd)[3] FdIII and the ferredoxin-NADP$^+$-oxidoreductase isoform II (FNRII) in maize chloroplasts in light compared with dark. Thus, diurnal changes in the thylakoids and/or the stromal redox system (*e.g.* the NADP$^+$/NADPH pool) seem to have an impact on the import characteristics of the organelle.

Taking this into account, it is not surprising that proteins with redox-active properties are also found at the stage of protein translocation, as is the case for the translocon at the inner envelope of chloroplasts, the Tic complex. Together with the protein import translocon present in the outer envelope of the chloroplast (the Toc complex) the Tic complex mediates the translocation of the vast majority of all nuclear-encoded pre-proteins destined for the chloroplast into the organelle (5–7). Seven Tic components have been described so far: Tic110, Tic40, Tic22, Tic20, and the three potentially redox-active components Tic62, Tic55, and Tic32. One of them, Tic55, had been found to co-purify with Tic110 in blue-native polyacrylamide gel electrophoresis (BN-PAGE) as well as in co-immunoprecipitations with Tic110 and precursor protein and represents a member of CAO/PAO-like oxygenases containing a Rieske-type [2Fe-2S] cluster and an additional mononuclear iron binding site (8). Another possible redox-acting component is Tic32, which had been identified as an interaction partner of the N-terminal domain of Tic110 and belongs to the conserved class of short chain dehydrogenases (SDRs, Ref. 9). Recently, the dehydrogenase activity of the recombinantly expressed protein was demonstrated *in vitro*, and it was found to be the target of Ca^{2+}/calmodulin (CaM)-regulation of protein import (10).

The third Tic protein with redox-active properties is Tic62, which had been found in close proximity to Tic110 and Tic55 in BN-PAGE (11) and consists of two distinct structural domains. While the N-terminal module features a pyridine nucleotide binding site (comparable to Tic32) and is conserved in all oxyphototrophic organisms down to green sulfur bacteria, the

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[3] The abbreviations used are: Fd, ferredoxin; Asc, ascorbic acid; At, *Arabidopsis thaliana*; CD, circular dichroism; CP, chloroplast; FNR, ferredoxin-NADP$^+$-oxidoreductase; HAR, hexammineruthenium trichloride; IE, inner envelope; Le, *Lycopersicon esculentum;* NBT, nitroblue tetrazolium; PC, phosphatidylcholine; SDR, short-chain dehydrogenase; Tic, translocon at the inner envelope of chloroplasts; Toc, translocon at the outer envelope of chloroplasts; CaM, calmodulin; NTA, nitrilotriacetic acid; BN-PAGE, blue-native PAGE.

C-terminal region is found only in vascular plants (12). It contains a series of unique Ser/Pro-rich repeats, which allow specific binding of Tic62 to FNR, a flavoenzyme best known for catalyzing the final step of the photosynthetic electron transport chain. FNR transfers electrons from Fd_{red} to $NADP^+$ generating NADPH, and thus reduces the chloroplast pool of redox equivalents. Interestingly, FNR is subject to a positional equilibrium within the chloroplast with a large amount of the protein also present in the stroma (13) as well as in the inner envelope (11). Upon oxidative stress, a re-localization takes place, which probably leads to a change in the electron distribution to pathways involved in chloroplast redox state maintenance such as *e.g.* the ascorbate peroxidase system or thioredoxins (14).

It was also reported that Tic62 dissociates from the Tic complex after addition of NADPH to isolated inner envelope vesicles (10). Together with its ability to bind pyridine nucleotides and its association with FNR, this finding would make Tic62 an ideal candidate to be involved in the redox regulation of the Tic complex. These considerations, therefore, led us to take a closer look at Tic62 and investigate its redox-dependent behavior as well as its interactions with FNR and the Tic complex in more detail.

We report here that Tic62 shuttles between the chloroplast membranes and the stroma, depending on the redox state of the plastidic $NADP^+$/NADPH pool. Oxidized conditions lead to a stronger attachment to the membrane as well as to the Tic complex, whereas reducing conditions favor a soluble localization in the stroma and association with FNR as an interaction partner. We demonstrate that the central amino acids (Ala-247 to Val-346) of Tic62 are sufficient for mediating binding to the Tic complex and that the N terminus with the predicted NADPH binding site is active as a *bona fide* dehydrogenase *in vitro*. The activity is specific for NADPH as electron donor and requires the presence of a lipid environment. Furthermore, we give the first experimental data on the structure of Tic62 derived from circular dichroism (CD) spectroscopic measurements of heterologously expressed and purified Tic62 constructs, demonstrating that the N and C terminus of Tic62 are not only functionally but also structurally different.

EXPERIMENTAL PROCEDURES

In Vitro Transcription and Translation—The coding region for PsTic62 from *Pisum sativum* either with or without the transit peptide was cloned into the vector pSP65 (Promega, Madison, WI) under the control of the SP6 promoter. Transcription of linearized plasmids was carried out in the presence of SP6 polymerase (MBI Fermentas, St. Leon-Roth, Germany). Translation was carried out using the Flexi Rabbit Reticulocyte Lysate System (Promega) in the presence of [^{35}S]methionine for radioactive labeling. After translation, the reaction mixture was centrifuged at 50.000 × *g* for 10 min at 4 °C, and the post-ribosomal supernatant was used for import experiments.

Chloroplast Isolation and Protein Import—Chloroplasts were isolated from the leaves of 9–11-day-old pea seedlings (*P. sativum* var. Arvica) and purified through Percoll density gradients as previously described (15, 16). A standard import reaction contained chloroplasts equivalent to 15–20 μg of chlorophyll in 100 μl of import buffer (330 mM sorbitol, 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% bovine serum albumin (w/v)), up to 3 mM ATP, and maximal 10% (v/v) ^{35}S-labeled translation products. Import reactions were initiated by the addition of chloroplasts to the import mixture and carried out for 15 min at 25 °C. Reactions were terminated by the separation of chloroplasts from the reaction mixture by centrifugation through a 40% (v/v) Percoll cushion. Chloroplasts were washed once, incubated with 20 mM HAR (hexammineruthenium trichloride) or ascorbic acid (Asc) for 10 min at 25 °C, ruptured in 25 mM Hepes/KOH, pH 7.6 for 30 min on ice, and separated into membrane and soluble fractions by centrifugation at 100,000 × *g* for 10 min at 4 °C. Import products were separated by SDS-PAGE, and radiolabeled proteins were analyzed by a phosphorimager.

Chloroplast Treatment and Fractionation—Isolated pea chloroplasts (20 μg of chlorophyll) were incubated with 0–20 mM oxidizing (HAR or oxidized glutathione GSSG) or reducing (Asc or reduced glutathione GSH) compounds for 20 min at 25 °C. For the rescue experiments, the HAR-treated chloroplasts were washed once in wash medium (330 mM sorbitol, 50 mM Hepes/KOH pH 7.6, 3 mM $MgCl_2$) and then treated with 20 mM of the reducing agents or incubated in ambient light for 20 min, while one sample was kept in the dark. In the reverse rescue, HAR treatment followed incubation with Asc. After subsequent washing, chloroplasts were disrupted in 25 mM Hepes/KOH, pH 7.5 by incubation on ice for 10 min, followed by centrifugation for 10 min at 100,000 × *g* or for 2 h at 600,000 × *g*, respectively. The pellet (membranes) and supernatant (stroma) were loaded completely and analyzed by SDS-PAGE and immunoblotting using antiserum against PsTic62 and PsTic110.

Overexpression and Purification of PsTic62-IA1, PsTic62-IA3, LeTic62 Full-length, LeTic62-Nt, and LeTic62-Ct—The constructs PsTic62-IA1 (IA1, Ala-247 to Leu-388) and PsTic62-IA3 (IA3, Val-346 to Ser-534) are described in Ref. 11. Use of the *Lycopersicon esculentum* (Le) constructs was necessary, because the corresponding ones from *P. sativum* (Ps, the full-length and the N-terminal domain, respectively) failed to express in the bacterial system presented here. The EST clone cLER19P1 was used as template for PCR. The mature LeTic62 full-length (Ala-1 to Ala-682), LeTic62-Nt (Ala-1 to Leu-260), and LeTic62-Ct (Gly-278 to Ala-682) constructs were cloned into pET21a vector using the restriction sites NheI/NotI, NdeI/XhoI, and NdeI/NotI, respectively. All constructs contained a C-terminal His_6 tag and were verified by DNA sequencing. For heterologous expression, the clones were transformed in *Escherichia coli* BL21 (DE3) cells and were grown at 37 °C in the presence of 100 μg/ml ampicillin to an A_{600} of 0.6. Expression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside, and cells were grown for 3 h at 37 °C (for PsTic62-IA1, PsTic62-IA3) or at 12 °C overnight (for LeTic62 full-length, LeTic62-Nt, and LeTic62-Ct), respectively. All proteins were purified via their C-terminal polyhistidine tags using Ni-NTA-Sepharose (GE Healthcare, Munich, Germany) under native conditions and eluted by the addition of 400 mM imidazole. The proteins were always used fresh and dialysed against 20 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 8.0, prior to CD analysis.

Co-immunoprecipitation of Tic62, Tic110, and FNR—Isolated pea chloroplasts (100 μg of chlorophyll) or inner envelope (IE) vesicles (50 μg of protein), isolated by sucrose density centrifugation (16), were incubated with oxidizing compounds (20 mM HAR for chloroplasts, 1 mM $NADP^+$ for inner envelope vesicles) or reducing agents (20 mM Asc for chloroplasts, 1 mM NADPH for inner envelope vesicles) for 20 min and solubilized with 1.5% decylmaltoside in 200 μl of IP buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl) for 10 min on ice. After centrifugation for 10 min at 100,000 × *g*, the supernatant was diluted 1:10 in IP buffer, 5 μl of primary antiserum (αPsTic62) were added and incubated for 2 h at room temperature, followed by subsequent incubation with 30 μl of protein A-Sepharose for 1 h at room temperature. The Sepharose beads were washed five times in IP buffer with 0.15% decylmaltoside and one time in IP buffer without detergent. Elution was performed using Laemmli sample buffer and incubation for 5 min at 95 °C. Load, Flow-through, washes, and elution fractions were analyzed by SDS-PAGE and immunoblotting using antisera against PsTic110 and AtFNR-L1. Quantification of the signals was performed using AIDA software (version 3.52.046).

Sucrose Gradient Sedimentation—250 μg of inner envelope vesicles were incubated with either H_2O, 1 mM $NADP^+$, or 1 mM NADPH for 15 min at 25 °C, followed by solubilization with 1.5% decylmaltoside for 15 min on ice. Sucrose gradients (10–50% w/v sucrose in 25 mM Hepes/KOH, pH 7.6, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, ±0.1 mM NADP(H)) were centrifuged at 342,000 × *g* for 16 h at 4 °C, and fractions of 200 μl were collected, precipitated with trichloroacetic acid, and analyzed by SDS-PAGE and immunoblotting using antiserum against PsTic62, PsTic110, and AtFNR-L1.

Dehydrogenase Activity Assay—Dehydrogenase activity assays were performed essentially as described (10). If not stated otherwise, 1 μg of purified protein (LeTic62 full-length, LeTic62-Nt, or LeTic62-Ct) was used, and the reaction was carried out in dehydrogenase buffer containing 10 mM Tris/HCl, pH 7.0, 100 μg (0.612 mM) nitroblue tetrazolium (NBT), and 100 μM NADPH in the presence of phosphatidylcholine (PC) liposomes (L-α-phosphatidylcholine type IV-S; Sigma; 1 mg/ml). Liposomes were generated by mixing 20 mg/ml L-α-phosphatidylcholine with 80 mM MEGA-9 (*N*-(D-glucityl)-*N*-methylnonanamide, *N*-nonanoyl-*N*-methylglucamide) in 10 mM Tris, pH 7.0, and subsequent dialysis against 10 mM KCl, 10 mM Tris, pH 7.0 overnight at 4 °C.

Binding Assays of Tic62 to FNR and the Tic Complex—The extraction of FNR from pea thylakoids and binding to the purified Tic62 constructs (PsTic62-IA1 and PsTic62-IA3) were performed as described (11). Briefly, FNR was enriched from thylakoids in the presence of 1 mM $NADP^+$ or NADPH, followed by incubation with 10 μg of expressed and purified Tic62 constructs for 2 h at room temperature. Tic62 polypeptides and interacting proteins were isolated by binding to Ni-NTA-Sepharose. Flow-through, washes, and elution fractions were analyzed by SDS-PAGE and immunoblotting.

For binding of Tic62 constructs (PsTic62-IA1, PsTic62-IA3, LeTic62-Nt, and LeTic62-Ct) to the Tic complex and FNR from stromal and membrane chloroplast fractions, pea chloroplasts were disrupted by incubation in 25 mM Hepes/KOH pH 8.0 for 10 min on ice and fractionated by centrifugation for 10 min at 100,000 × *g*. The pellet (membranes) was solubilized in 50 mM Tris/HCl, pH 8.0, 150 mM NaCl with 1.5% decylmaltoside for 10 min on ice, centrifuged for 10 min at 100,000 × *g* at 4 °C, and both the solubilized membranes and the supernatant after fractionation (stroma) were incubated with 10 μg of the respective Tic62 constructs for 2 h at room temperature. The proteins were subsequently isolated by binding to Ni-NTA-Sepharose overnight at 4 °C. Flow-through, washes, and elution fractions were analyzed by SDS-PAGE and immunoblotting.

Circular Dichroism Measurement—Circular dichroism experiments using LeTic62-Nt and PsTic62-IA3 were carried out at room temperature using a Jasco J-810 spectropolarimeter flushed with nitrogen. Spectra were collected from 260 to 185 nm using a 1-mm pathlength of a cylindrical quartz cell. Each spectrum was the average of three scans taken at a scan rate of 50 nm/min with a spectral bandwidth of 1 nm. The concentration of proteins varied from 0.082 to 0.284 mg/ml. Where indicated, 0.01–0.1 mg/ml sonicated PC liposomes or 50 μM NADP(H) were added and incubated for 15 min at room temperature with protein prior to the measurement. For the final representation, the baseline was subtracted from the spectrum. Experiments were done in duplicate or triplicate. The analysis was performed using the CDSSTR program (protein reference set 3) from the DichroWeb server (17) or the CDNN program (CD Spectra Deconvolution Version, 2.1).

RESULTS

Tic62 Shuttles between Chloroplast Membranes and Stroma—Chloroplasts subdivided into membrane and stromal fraction by ultracentrifugation at 100,000 × *g* show an approximately equal distribution of Tic62 in both compartments (Fig. 1*A*). Higher centrifugation speeds (up to 600,000 × *g* for 2 h) did not alter this distribution, which excludes the possibility that Tic62 stays in the supernatant because of residual membrane shreds (as demonstrated for Toc159 in Ref. 18; data not shown). Because Tic62 was suggested to be a possible redox sensor protein (11), we were interested in answering the question of whether the localization of Tic62 might itself be influenced by redox conditions. Incubation of chloroplasts with oxidizing or reducing agents clearly changed this distribution (Fig. 1*A*). After treatment with HAR, a water-soluble electron acceptor that oxidizes the NADPH pool in the stroma (19), Tic62 was found to be more associated with the membrane fraction in a concentration-dependent manner. Similar effects were observed upon incubation with GSSG, the oxidized form of glutathione. In contrast to this, treatment of chloroplasts with reducing agents like Asc or the reduced form of glutathione (GSH) showed a slightly higher accumulation of the protein in the stroma, even though the effect was not as drastic as observed with oxidizing compounds. This suggests that a subpool of Tic62 remains membrane-bound *in vitro*. Tic110, used as an inner envelope control protein, showed no difference in distribution upon treatment with oxidizing or reducing agents (Fig. 1*A*). Furthermore, incubation of mildly solubilized (0.15% decylmaltoside) inner envelope vesicles with NADPH led to an increase of the Tic62 amount in the supernatant compared to incubation with $NADP^+$ or H_2O (data not shown). Although

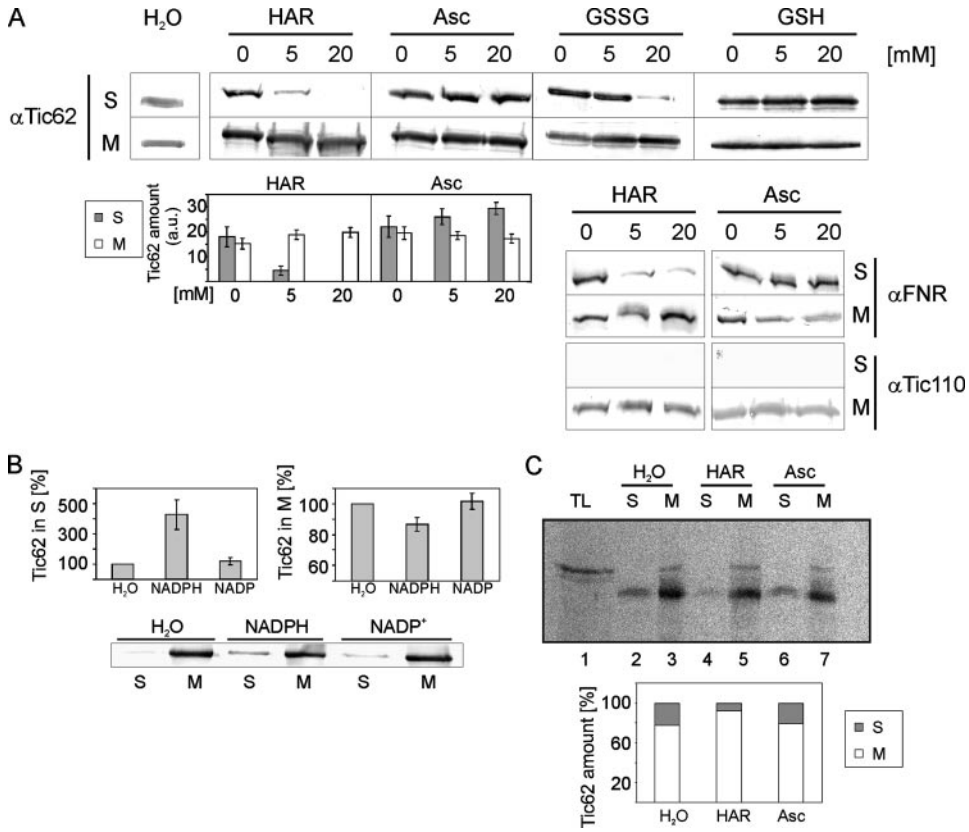

FIGURE 1. **The distribution of Tic62 in the chloroplast is dependent on the redox state.** *A*, isolated pea chloroplasts were incubated for 20 min with oxidizing agents (HAR, GSSG), reducing agents (Asc, GSH), or H_2O as a control, followed by disruption and separation into membranes and soluble fraction. The samples were analyzed by immunoblot with αTic62, αTic110, or αFNR. Standard error bars are included in the quantifications. *B*, inner envelope vesicles (20 μg of protein) were mildly solubilized in 0.15% decylmaltoside and treated with 1 mM $NADP^+$, NADPH, or water as a control in the presence of 1 μg of FNR. The presence of Tic62 in supernatant and pellet fractions after centrifugation for 10 min at 100,000 × *g* was analyzed by immunoblotting. Standard error bars are included in the quantifications. *C*, phosphorimage of import of Tic62 into isolated pea chloroplasts and quantification of import. *In vitro* translated PspreTic62 was imported for 10 min in the presence of 3 mM ATP. After import, chloroplasts were treated with H_2O, HAR, or Asc, respectively and soluble and membrane samples were obtained. *S*, soluble fraction; *M*, membranes; *TL*, 10% of translation product.

stroma and a total chloroplast membrane fraction in untreated control chloroplasts (Fig. 1*A*).

To address the question of whether the observed attachment of Tic62 to the membrane fraction under oxidizing conditions is reversible and therefore of physiological relevance in the chloroplast, we investigated a possible re-localization of Tic62 back to the stroma after changing the incubation buffer from oxidizing to reducing conditions. Indeed, HAR-induced membrane binding was found to be reversible. When chloroplasts were treated with HAR for 10 min, followed by washing and incubation with Asc or GSH, a re-localization of Tic62 to the stroma was observed (Fig. 2*B*). Moreover, even incubation of HAR-treated chloroplasts for 20 min in ambient light, which resulted in reinitiation of photosynthetic electron transport and production of NADPH, led to a slight, but noticeable re-shuttling of Tic62 into the stroma. A time-dependent analysis of this rescue showed that the re-localization of Tic62 induced by Asc was slower than the initial membrane binding caused by oxidizing agents (Fig. 2*C*). This could be explained by (i) the strong NADPH oxidizing effect of HAR, which might not be washed out completely or (ii) by a decreasing viability of chloroplasts after the incubation steps. Furthermore, in a reverse rescue experiment, the insertion of Tic62 into the inner membrane could be induced if HAR treatment followed Asc incubation (Fig. 2*D*). In this approach, chloroplasts were first treated with 20 mM Asc and subsequently incubated with HAR, which induced a reassociation of Tic62 with the membrane. Thus, the relocalization of Tic62 even *in vitro* suggests a physiological role of the observed Tic62 shuttling and a highly dynamic distribution of the protein, which is strictly dependent on the redox state of the chloroplast. A mere unspecific aggregation of Tic62 seems therefore unlikely.

this effect was much weaker than the observed shuttling in intact chloroplasts, it could be slightly enhanced (~1.5-fold) by the addition of FNR (Fig. 1*B*). As experiments with HAR/GSSG and Asc/GSH consistently resulted in similar observations, only the results obtained with HAR and Asc are described below.

A similar redox-dependent membrane association of Tic62 was also observed when the distribution of newly imported protein was analyzed (Fig. 1*C*). After import of the protein for 15 min, reisolation, and subsequent treatment of the chloroplasts with HAR, we observed very small amounts of Tic62 in the soluble fraction in contrast to the control reaction with H_2O or Asc (compare *lanes 2*, *4*, and *6*). A more detailed analysis of the HAR-induced membrane association of Tic62 revealed that this effect occurred very rapidly (Fig. 2*A*). After 1 min of incubation with HAR, the majority of Tic62 was found in the membrane fraction, while the distribution of Tic110, used as inner envelope control protein, did not change. Moreover, we detected that FNR reacted similarly to changes of the chloroplast redox state: *e.g.* upon treatment of chloroplasts with HAR, the attachment of this protein to the membrane fraction increased markedly, whereas it was equally distributed between

The Interaction of Tic62 with the Tic Complex and FNR Is Redox-dependent—The co-localization and interaction of Tic62 with the Tic complex had been investigated previously (11, 20). In these studies, Tic62 was described as part of the Tic complex, but it was suggested that the association of Tic62 with Tic110 might be quite dynamic. A prominent feature of Tic62 is its strong interaction with FNR via the repetitive motif in the C terminus (11). Several Tic62 constructs were used to investigate the role of these repeats in the binding to FNR: IA1, located in the middle part of PsTic62 (containing no FNR-interacting

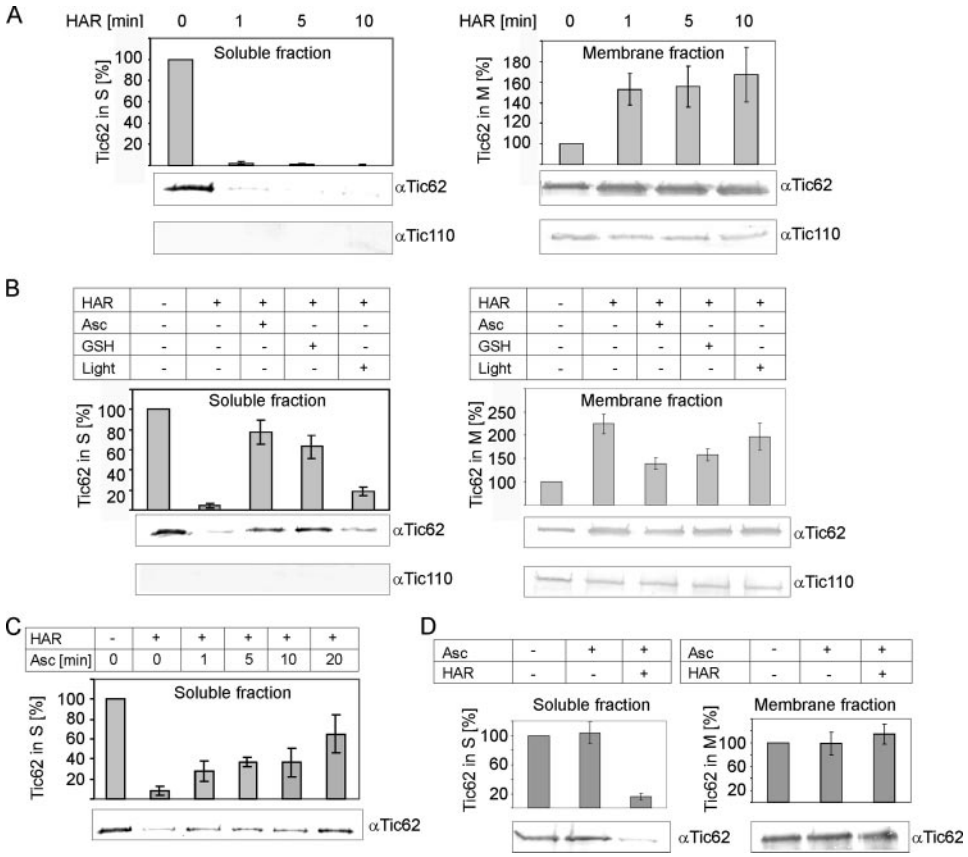

FIGURE 2. **Characterization of the observed Tic62 shuttling.** *A*, time course of HAR treatment. Incubation of chloroplasts with HAR was stopped after the indicated time points and chloroplasts were lysed and fractionated. The samples were analyzed by immunoblot with αTic62 and αTic110, soluble and membrane fractions are depicted. *B*, rescue of HAR treatment with reducing agents. HAR-incubated chloroplasts were either kept in the dark, incubated with reducing compounds (Asc, GSH), or kept in the light for 20 min, followed by separation into soluble fractions and membranes. The presence of Tic62 and Tic110 in the soluble and pellet fractions is shown by immunoblotting. *C*, time course of rescue. Pea chloroplasts were treated with HAR for 10 min, washed, and incubated with Asc for the time indicated. The samples were analyzed by immunoblot with αTic62. *D*, reverse rescue. Pea chloroplasts were first treated with Asc for 10 min, washed, and subsequently incubated with HAR for 5 min. The samples (supernatant and pellet after fractionation) were analyzed by immunoblot with αTic62. Standard error bars are included in the quantifications. All experiments were performed in duplicate or triplicate. *S*, soluble fraction; *M*, membranes.

repeats) and IA3, containing all three repeats from the C terminus (Fig. 3*A*). The same constructs were also used in the present study to analyze the influence of different redox environments on the interaction of Tic62 and FNR in the presence or absence of $NADP^+$ or NADPH, respectively.

To test whether the FNR was specifically pulled-down in our experimental system, we first used the same setup as described (11). When the expressed and purified PsTic62 constructs IA1 and IA3 were incubated with FNR, it was only detectable in the presence of IA3, but not with IA1, as reported before (Fig. 3*A*, *control reaction*). After adding pyridine nucleotides to the reaction buffer, and thus mimicking changes in the redox status during the incubation, we found this interaction to be strongly dependent on redox conditions. Incubation of the samples with $NADP^+$ reduced the interaction between FNR and IA3, whereas NADPH led to a marked increase (Fig. 3*A*, compare *lanes 3* and *6* of the control reaction). Similar results were obtained by co-immunoprecipitation experiments (Fig. 3*B*). Isolated chloroplasts or inner envelope vesicles were preincubated with oxidizing (HAR for chloroplasts, $NADP^+$ for inner envelopes) or reducing (Asc for chloroplasts, NADPH for inner envelopes) compounds, followed by co-immunoprecipitation with αTic62 antiserum. Again, the interaction of Tic62 with FNR was stronger under reducing conditions (increase of 40 and 24%, respectively) and weaker when oxidizing agents were used (decrease of 35 and 29%, respectively; Fig. 3*B*). Interestingly, the opposite effect was observed for the interaction of Tic62 with Tic110. A close proximity of these two proteins had been described before, when Tic62 was found to co-migrate with Tic110 in BN-PAGE (11). Furthermore, antibodies against Tic62 and Tic110 efficiently co-immunoprecipitated the other respective component. We were now able to show that this interaction is disrupted in a reduced redox environment and clearly enhanced under oxidizing conditions (Fig. 3*C*). This effect occured both in isolated chloroplasts and inner envelope vesicles, but was found to be most prominent in whole chloroplasts, where preincubation with HAR resulted in a ~4-fold increase of the interaction (Fig. 3*C*).

To verify our results from the pull-down assays by another experimental approach, we investigated the redox-dependent co-localization of Tic62 with Tic110 and FNR by sucrose density centrifugation of solubilized inner envelope vesicles. The vesicles where preincubated with either $NADP^+$, NADPH or water as control, solubilized, and the migration of proteins was analyzed after centrifugation to equilibrium on linear sucrose density gradients (Fig. 4). In untreated or $NADP^+$-treated inner envelope vesicles, Tic62 was found to co-localize with Tic110. In contrast, preincubation with NADPH resulted in a shift of Tic62 toward lower density fractions and a co-localization with the FNR. Taken together, these results suggest that not only the distribution, but also the interaction of Tic62 with its partner proteins is redox-dependent. Binding of Tic62 to the Tic complex at the inner envelope membrane is enhanced under oxidizing conditions, whereas the protein dissociates from the complex under reducing conditions, leading to a preferred interaction with the FNR.

Tic62 Has Dehydrogenase Activity—The Tic complex contains three components possibly involved in redox-regulation of protein import: Tic55, a Rieske iron-sulfur protein, Tic32 and Tic62, both grouped into the family of SDRs according to sequence homologies of the NADPH-binding sites (9, 12). The dehydrogenase activity of Tic32 had been experimentally

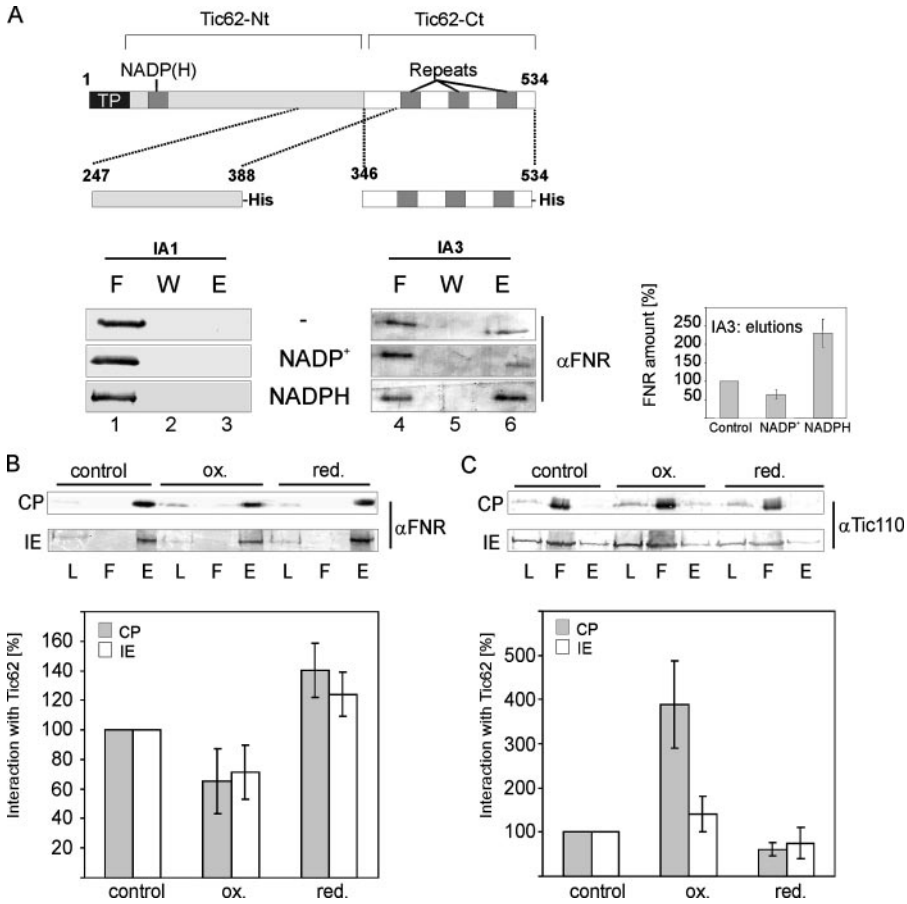

FIGURE 3. **Oxidizing or reducing conditions influence the interactions of Tic62 with the Tic complex and FNR.** *A*, schematic overview of the Tic62 constructs used in this study. For investigation of the redox-dependent interaction of Tic62 with FNR, FNR was isolated from pea thylakoids and incubated with His-tagged PsTic62 proteins, containing either none or all three FNR-interacting repeats (IA1 and IA3, respectively; Ref. 11), after adding H_2O or 1 mM $NADP^+$ or NADPH to the samples, proteins were pulled-down using Ni-beads, and the presence of FNR was investigated by immunoblot. *Lanes 1* and *4*, flow-through; *lanes 2* and *5*, wash; *lanes 3* and *6*, elutions. Quantification of the elution fractions is also depicted. Experiments were performed in triplicate. Co-immunoprecipitation experiments show the interactions of Tic62 with either FNR (*B*) or Tic110 (*C*). Isolated pea chloroplasts or inner envelope vesicles were treated with either water as control, or with oxidizing (HAR for chloroplasts, $NADP^+$ for inner envelope) or reducing (Asc for chloroplasts, NADPH for inner envelopes) compounds, solubilized with 1.5% decylmaltoside, and co-immunoprecipitated with αTic62. The presence of FNR and Tic110 was tested by immunoblotting. 1:20 (for chloroplasts) or 1:10 (for inner envelopes) of the load, 1:20 of the flow-through, and total elution fractions were analyzed. Standard error bars are included in the quantifications. Normalization was performed by arbitrarily setting the value of the control to 100% and calculating the amount of Tic62 in the samples incubated with oxidizing/reducing components relative to the control. All experiments were done in triplicate. *L*, load; *F*, flow-through; *W*, wash; *E*, elution; *CP*, chloroplasts; *IE*, inner envelope vesicles.

shown recently (10) using the artificial electron acceptor NBT. In the assay, a reduction of NBT to formazan is easily visible by the appearance of a lilac precipitation. Using a similar assay, we investigated the dehydrogenase activity of heterologously expressed and purified Tic62. Because the constructs from *P. sativum* failed to express, we decided to use the orthologous protein from *L. esculentum*. LeTic62 also belongs to group I of the Tic62 protein family (Balsera *et al.* (12)), which contains the original Tic62 proteins that possess the repetitive modules for interaction with the FNR, a transit peptide for targeting to the chloroplast and which are proposed to be localized in the inner envelope membrane. The N-terminal domains of LeTic62 and PsTic62, including the dehydrogenase site, are highly conserved and contain about 76% identical amino acids. Differences are only observed in the C-terminal domains, where LeTic62 has seven FNR-binding repeats in contrast to three in PsTic62. Nevertheless, the sequence of these repetitive modules was found to be extremely conserved as well (an alignment of both proteins is shown in Fig. 5*A*). For investigating the Tic62 dehydrogenase activity, we analyzed not only the full-length LeTic62 protein, but also the N- and C-terminal domains, respectively. An NADPH-dependent reduction of NBT to formazan was observed for the full-length protein and LeTic62-Nt, containing the NADPH-binding site, but not for LeTic62-Ct (Fig. 5*B*, *upper panel*). Strikingly, dehydrogenase activity was only detectable after preincubation of Tic62 with lipids, a feature that was also discovered for Tic32 (10). No reduction of NBT was observed in the absence of Tic62 or lipids, and NADH could not substitute for NADPH in this assay. Heat inactivation of the purified protein abolished formazan formation (Fig. 5*B*, *lane H*). Furthermore, the dehydrogenase activity was found to be dependent not only on the concentration of Tic62, but also of NADPH, the substrate NBT, and the reaction time (Fig. 5, *C* and *D*). Together, these data provide evidence that Tic62 is a *bona fide* member of NADPH-dependent dehydrogenases, and that its properties (NADPH specificity and requirement of a lipid environment) are similar to Tic32.

Structural Analysis of Tic62—Two previous publications (11, 12) and the data from this study suggest that the N- and C-terminal modules of Tic62 have distinct functions. It is therefore likely that their structures also differ considerably. To gain experimental evidence for this prediction, we performed circular dichroism measurements of parts of both modules (LeTic62-Nt and PsTic62-IA3; Fig. 6). The CD data obtained for Tic62-Nt as analyzed by the CDSSTR software from DichroWeb (17) indicated a structure consisting of roughly 28% α-helices, 21% β-sheets, and 19% turns (Fig. 6*A*). In contrast, PsTic62-IA3 (C terminus of Tic62) revealed a disordered structure possibly involving poly (Pro) II helix features (Fig. 6*B*; analyzed according to Kelly *et al.* (21)).

In view of the lipid-dependent dehydrogenase activity, we performed CD spectroscopy of the N terminus in the presence of sonicated PC liposomes (Fig. 6*A*). However, no obvious changes of the secondary structure could be observed upon

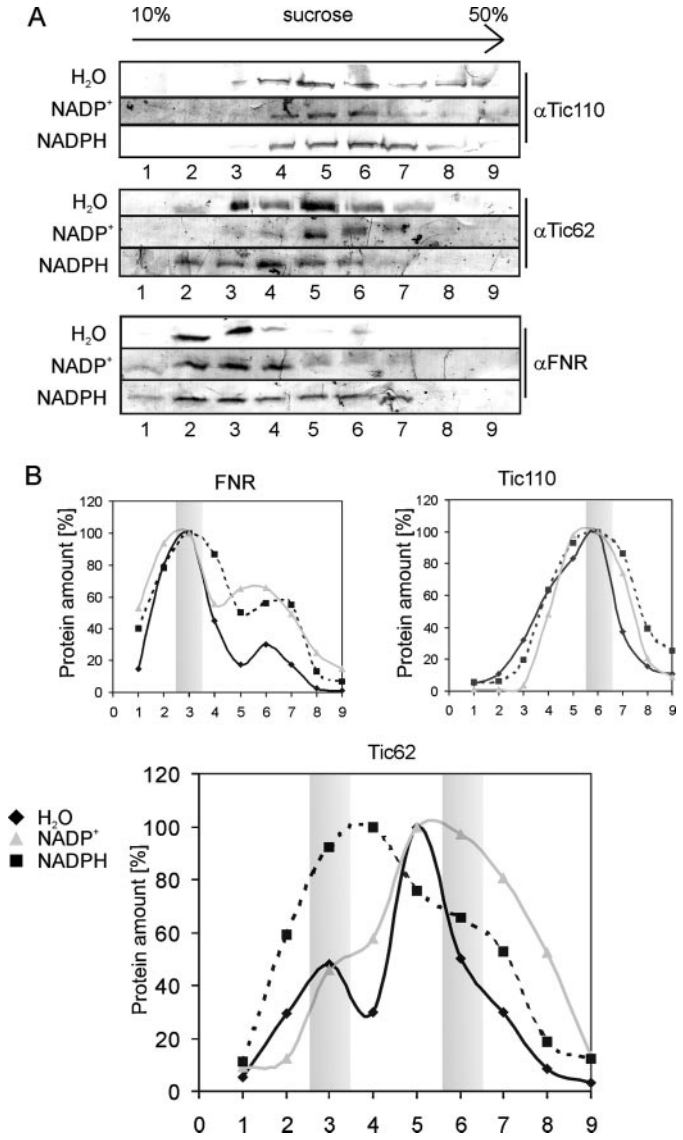

FIGURE 4. **Co-localization of Tic62 with either Tic110 or FNR in sucrose gradients is redox-dependent.** Inner envelope vesicles were incubated with 1 mM $NADP^+$, NADPH, or H_2O as control, solubilized with 1.5% decylmaltoside and loaded on linear sucrose gradients. The presence of Tic110, Tic62, and FNR was analyzed in the samples (0, lowest density; 9, highest density). *A*, immunoblots with αTic110, αTic62, and αFNR of one experiment and *B*, quantifications of this experiment are shown. For the quantifications, the highest value of each blot was arbitrarily set as 100%, and the other values were calculated proportionally. Sucrose gradient analyses were performed five times; a typical result is shown.

addition of liposomes in our experimental setup. We furthermore analyzed LeTic62-Nt in the presence of $NADP^+$ and NADPH. Interestingly, incubation of the protein with these pyridine nucleotides prior to CD measurements caused a clear difference in the observed spectra. Especially the helical content of Tic62, as judged by the mean residue ellipticity at 222 nm (Θ_{222}), was altered by the presence of $NADP^+$ (and less by NADPH; Θ_{222}(Tic62-Nt) = −7951.51; Θ_{222}(Tic62-Nt + $NADP^+$) = −9019.75; Θ_{222}(Tic62-Nt + NADPH) = −8157.50). This indicates a conformational change of Tic62 taking place upon binding to NADP(H) (Fig. 6*C*). Spectra analyses showed an increase of ~2–3% α-helices in the presence of $NADP^+$ with a concomitant decrease of random coils (~2%). Altogether, the recorded spectra corroborate the predictions and demonstrate that Tic62 is made up of two structurally and functionally different domains.

Characterization of Tic62 Binding to the Tic Complex—The experiments presented so far clearly demonstrate that the N- and C-terminal modules of Tic62 are structurally and functionally very different. Based on these findings we wanted to know whether the binding to the Tic complex can also be ascribed to one of the two modules alone, or whether both domains function in a concerted action. To this end, we performed a binding assay employing several His-tagged Tic62 constructs (as described in Fig. 3). Solubilized chloroplast membranes were incubated with the heterologously expressed and purified Tic62 constructs, and the presence of Tic110, as a marker protein for the Tic complex, was analyzed by immunoblots after pull-down with Ni-beads (Fig. 7). No Tic110 was detected in elutions of samples without added His-tagged Tic62 proteins, which excludes an unspecific binding of Tic110 to the Ni^{2+} matrix (Fig. 7, *control*). However, Tic110 was found in the elution fractions after pull-down with LeTic62 (full-length), LeTic62-Nt, and PsTic62-IA1, but not with PsTic62-IA3, representing the C-terminal part of the protein (Fig. 7). These findings imply that parts of the N terminus as well as of the middle part are able to mediate interaction with the Tic complex, and that the central domain of Tic62, containing determinants of both the N and the C terminus, is sufficient for the binding.

DISCUSSION

To be able to sustain a functional electron transfer chain and a stable redox homeostasis in photosynthetically active chloroplasts, the relative abundance as well as the activity of all proteins participating in these processes have to be tightly controlled. However, most proteins involved in photosynthesis and the reductive downstream reactions, and all enzymes involved in the response to oxidative stress are nuclear-encoded. Thus, deviations from the usual redox state have to be sensed, and the signal has then to be faithfully transduced to the cell nucleus. For optimal performance of the system, regulation therefore has to take place at several stages and in several compartments, *i.e.* transcription and translation in the nucleus and cytoplasm, respectively, and activation/inactivation as well as degradation of the enzymes involved inside the organelle. Research in these areas has unraveled a complex network in recent years (1, 22). In addition, the discovery of three putatively redox-acting proteins associated with the Tic complex points at yet another hitherto unknown checkpoint in this network being present at the stage of protein translocation.

In this study, Tic62 was analyzed in more detail. Because it had already been proposed to act as a redox sensor due to its ability to bind pyridine nucleotides and interact with the FNR we strived to answer several questions arising from previous observations: is Tic62 subject to redox regulation itself and, if so, whether this has an influence on the association with its interaction partners. Additionally, the question of whether Tic62 is an active dehydrogenase was investigated.

Redox-dependent Shuttling of Tic62 between the Envelope Membrane and the Stroma—Artificial oxidation or reduction of the $NADP^+$/NADPH pool in the chloroplast resulted in a

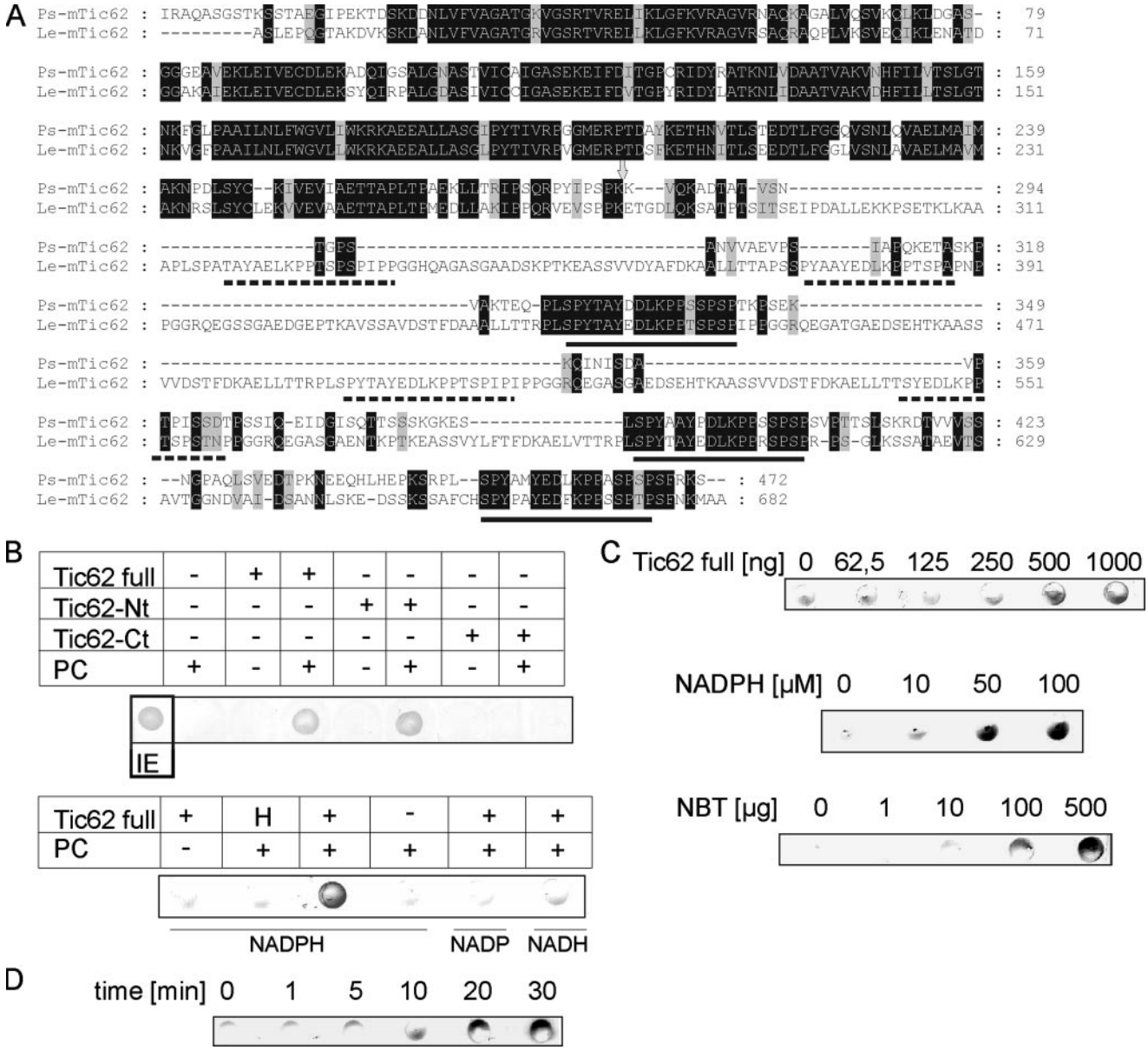

FIGURE 5. **Tic62 is an active NADPH-dependent dehydrogenase.** An alignment of LeTic62 and PsTic62 is depicted in *A*. An *arrow* indicates the end of the conserved N-terminal part, *solid lines* mark the FNR-interacting repeats present in both proteins, *dashed lines* represent the repeat motifs only found in LeTic62. *B*, *upper panel*, 1 μg of protein (LeTic62 full-length, the N-terminal, or the C-terminal part) was incubated for 30 min at 25 °C with or without liposomes, spotted on a nitrocellulose membrane and incubated in dehydrogenase buffer, containing 100 μM NADPH, 10 mM Tris, pH 7.0, and 100 μg of NBT. Reduction of NBT to formazan becomes visible by a lilac precipitation. 1 μg of inner envelope was used as a positive control. *B*, *lower panel*, $NADP^+$ or NADH could not substitute for NADPH in this assay, and preincubation of the protein at 95 °C (H) for 5 min abolishes dehydrogenase activity. *C*, concentration dependence of Tic62, NADPH, and NBT. Samples were incubated in the respective buffer for 20 min each. *D*, time dependence of dehydrogenase activity with 1 μg of protein, 100 μM NADPH, and 100 μg of NBT. *H*, heat inactivation; *PC*, phosphatidylcholine.

remarkable relocation of Tic62 between the membrane fraction and the stroma (Fig. 1*A*). The observed effect clearly demonstrates that Tic62 is able to react to changing redox conditions and indicates that important properties of the protein itself are redox-regulated. Alterations in protein localization after perception of a redox signal is a known feature also described for other proteins: the FNR itself *e.g.* was found to change its distribution in the chloroplast because of oxidative stress (14). In this study, addition of methyl viologen or H_2O_2 led to a solubilization of the FNR from the thylakoids to the stroma. This seems to contradict the findings presented here, where the attachment of the FNR to the membrane fraction increased after treatment with oxidizing compounds. It has to be taken into account, however, that the effect of HAR used in the present study differs considerably from the effects caused by methyl viologen and H_2O_2. HAR is a water-soluble electron acceptor that supports a high rate of pyridine nucleotide oxidation and therefore selectively oxidizes the NADPH pool in the stroma (11, 19). In contrast to this, methyl viologen catalyzes the photoreduction of O_2 at photosystem I, accelerating the production of O_2^- and H_2O_2 (23, 24). The accumulation of H_2O_2 subsequently leads to oxidative stress and the inactivation of several important enzymes such as fructose-1,6-bisphosphatase, NADP-glyceraldehyde-phosphate dehydrogenase, ascorbate

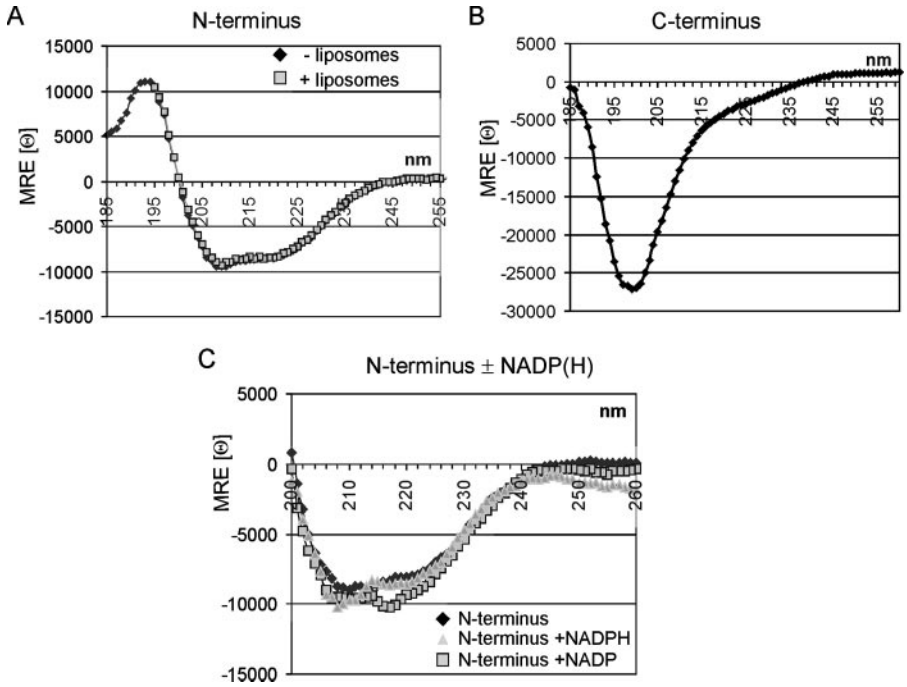

FIGURE 6. **Structural analysis of Tic62 constructs.** Circular dichroism spectroscopy of the Tic62 N terminus with and without addition of sonicated PC liposomes (LeTic62-Nt, *A*) and of the C terminus (PsTic62-IA3, *B*) and the N terminus with and without 50 μM $NADP^+$ or NADPH (LeTic62-Nt, ±NADP(H), *C*). The analysis of one typical spectrum (an average of three scans with a spectral bandwidth of 1 nm) is shown. Data were converted to mean residue ellipticity (MRE [Θ] in degrees cm^2 $dmol^{-1}$ $residue^{-1}$).

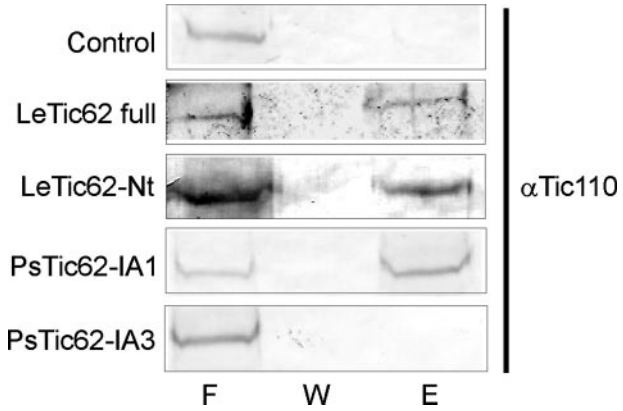

FIGURE 7. **Determination of the Tic complex binding site of Tic62.** Isolated pea chloroplasts were disrupted in 25 mM Hepes/KOH pH 7.6 and fractionated into soluble fraction and membranes. The membranes were solubilized with 1.5% decylmaltoside and incubated with either H_2O as control, purified LeTic62 full-length, LeTic62-Nt, PsTic62-IA1, and PsTic62-IA3, respectively. After affinity purification of the proteins with Ni-beads, the presence of Tic110 was analyzed by immunoblots. *F*, flow-through; *W*, wash; *E*, elution.

peroxidase, and superoxide dismutase. These differing effects of HAR on the one and methyl viologen/H_2O_2 on the other side might offer an explanation for this discrepancy.

In an earlier study, Tic62 was found to be largely carbonate-resistant in IE vesicles, whereas it showed some sensitivity to urea extraction (Ref. 11 and Fig. 2*D*). This observation could very well be explained by the presence of the hydrophobic patch on the surface of the N-terminal module proposed by Balsera *et al.* (12). Whereas treatment with high pH is known to disrupt protein-protein interactions, the bilayer stays intact, and protein-lipid interactions usually remain. Only when structural features were destabilized by incubation with urea, a partial dissociation into the supernatant was observed (Ref. 11 and Fig. 2*D*), corroborating the model/hypothesis presented in this study. The proposed shuttling model for Tic62 clearly furthers the data and conclusions presented by Küchler *et al.* (11), who primarily observed a membrane-bound location for Tic62, which could have resulted from the experimental setup (see below). Furthermore, it has to be taken into account that purified stroma and envelope vesicles were used for the early experiments on the Tic62 localization rather than whole chloroplasts as in this study. The lengthy procedure necessary for the envelope and stroma isolation promotes oxidation of the proteins, which could explain in part the low abundance of Tic62 observed in the stromal fraction. Additionally, the loading of equal protein amounts of chloroplast fractions (as done by Küchler *et al.* (11) in Fig. 2*B*) leads to an under-representation of the stromal signals relative to the envelopes, which constitute about 1% of total chloroplast proteins, whereas the stroma represents ~50%.

The fact that the membrane binding of Tic62 was found to be reversible upon re-reduction of the NADP(H) pool by adding *e.g.* Asc or by incubating the chloroplasts in the light (Fig. 2, *B* and *C*), points to a physiological role of Tic62 shuttling. The observed redox-dependent behavior fits very well to its predicted function as a chloroplast redox sensor. In this way, information about the metabolic redox state of the chloroplast could potentially be transmitted to the translocon at the inner envelope.

The Interactions of Tic62 with the Tic Complex and FNR Are Redox-regulated—Interestingly, we observed that the redox-dependent shuttling of Tic62 correlated tightly with a change in its interaction partners: under oxidizing conditions, not only the attachment of Tic62 to the membrane fraction was increased, but also its binding to the Tic complex. In contrast, reducing conditions caused less interaction of Tic62 with the Tic complex and a stronger attachment to FNR (Fig. 3, *B* and *C*). Most of the observed changes, although reproducible, were small, because only ~20–30% of the interactions with the respective partner protein were affected. An exception from this was observed in intact chloroplasts, where incubation with HAR led to a ~4-fold increase of the interaction of Tic62 with Tic110 (Fig. 3*C*). It is therefore likely that the observed membrane binding of Tic62 is more prominent *in vivo* than it is detectable in our *in vitro* system.

We conclude that both, the attachment of Tic62 to the Tic complex as well as the binding to FNR, are important properties

of Tic62 function, and propose that changes in the metabolic redox conditions, and thus in Tic62 distribution, might be involved in a fine-tuning or optimization of downstream events, as *e.g.* protein translocation.

The data presented in this study imply that the interaction of Tic62 with the Tic complex is highly dynamic, because reducing conditions cause Tic62 to dissociate from the Tic translocon. A similar effect had been observed before, where the interaction of Tic32, the second NADPH-dependent dehydrogenase in the Tic complex, with Tic110 was influenced in a similar manner (10). Therefore, reducing conditions in the chloroplast could cause a transient disassembly of redox-acting components from the Tic complex. As our current working hypothesis we propose that this change of translocon composition might result in a change in the import efficiency of a specific subset of precursor proteins, which are necessary for the chloroplast only in certain conditions directly related to the metabolic redox state as *e.g.* oxidative *versus* reductive biosynthetic pathways. However, whether the presence and/or absence of Tic62 has any effect on import and which precursor proteins might be affected remains to be investigated.

Küchler *et al.* (11) reported that the binding of FNR to Tic62 is mediated by a unique repetitive motif in the C-terminal part of Tic62. The repeats are enriched in proline and serine residues and were found to be specific for the interaction with FNR. In contrast, no binding site to the Tic complex had been described as yet. Making use of a series of deletion constructs in pull-down experiments, we were now able to determine the location of the binding site in some detail (Fig. 7). Knowing that the full-length LeTic62 protein is able to interact with the Tic complex *in vivo* (Fig. 7), we first analyzed the N- and C-terminal modules independently of each other. Examining the N-terminal construct (LeTic62-Nt) in the assay, we found that the C-terminal deletion did not abolish binding to the Tic complex. The use of PsTic62-IA3, comprising the C terminus with the FNR-binding repeats, however, established further that this region seems not to be involved in the interaction with the Tic complex but probably is specific for the interaction with FNR (see Fig. 3*A*). Finally, we used a third small (~15 kDa) Tic62 fragment (PsTic62-IA1) located in the center of the protein, which was sufficient for binding to the Tic complex (Fig. 7). Based on these findings, we conclude that the interaction site to the Tic complex seems to be located in the central domain of Tic62 (amino acids Ala-247 to Val-346 of the pea sequence). This corroborates the proposed theory that only by the fusion of the evolutionary unrelated N- and C-terminal modules was this binding site created and, thus, led to the appearance of the Tic62 class of proteins (12).

Structural Investigations of Tic62—It was proposed that Tic62 consists of two different modules, which have not only an independent origin, but also different functions (12). The N-terminal part containing the NADP(H) binding site is evolutionary very ancient and highly conserved from higher plants to cyanobacteria and even green sulfur bacteria. The C terminus, on the other hand, with the proline/serine-rich FNR-interacting repeats was found to be unique and probably became part of the protein only recently in evolution, because the full-length Tic62 protein including the C-terminal repeats is found only in vascular plants. Information on the putative structure of the Tic62 N terminus was already available using computational modeling with a homolog to the N terminus as template (12). Structural determination by CD spectroscopy now revealed that both domains differ markedly in the observed structures of the purified fragments (Fig. 6, *A* and *B*). Analysis of the N terminus roughly confirmed the predicted structure from the model (~31% α-helices, ~26% β-sheets; Ref. 12). In contrast to this, the C terminus was found to contain mostly disordered structures and some features that might be interpreted as poly (Pro) II helices, which fits with the high Pro content of the C-terminal repeats. The disordered structure of the C terminus leads us to propose that binding to FNR is necessary to gain an ordered three-dimensional structure in this part of the protein, because it is known that disordered segments often fold on binding to their biological targets. Moreover, disordered regions are often functional and highly conserved (25).

We were furthermore interested in a possible conformational change of Tic62 in a lipid environment or upon binding to NADP(H). Liposome binding assays showed that radioactively labeled mature Tic62 was able to bind to liposomes and thermolysin-digested inner envelope vesicles *in vitro* (data not shown). This binding is presumably mediated by the N terminus because dehydrogenase activity was observed in this domain after the addition of liposomes (Fig. 5*B*). The hydrophobic patch described earlier could permit the attachment to the membrane. However, when sonicated liposomes were added to the LeTic62-Nt in CD experiments, our data indicate that the binding of the N terminus to liposomes proceeds without obvious changes in the secondary structure of Tic62 (Fig. 6*A*) in our experimental setup (far UV CD spectroscopy), although this does not exclude changes in Tic62 conformation. Further experiments have to be performed to investigate this issue in more detail. Nevertheless, when $NADP^+$ or NADPH was added to LeTic62-Nt prior to CD measurements, the observed spectra differed noticeably from protein without additional cofactors. This change was particularly prominent when $NADP^+$ was added (Fig. 6*C*), which led to a ~2–3% increase of α-helices. We conclude that a conformational change takes place in Tic62 upon binding of pyridine nucleotides. It has to be noted that $NADP^+$ causes a different change compared with NADPH addition. This effect might offer an explanation for the observed Tic62 shuttling between the membranes and the stroma, as the hydrophobicity of the protein could be altered by the cofactor. This observation might explain the attachment to or dissociation from the membrane.

Taking together the findings of our structural investigations, we would like to extend the current model of Tic62 and propose the existence of four functional regions distributed over the two structurally distinct modules: (i) the NADP(H) binding site in the extreme N terminus, possessing dehydrogenase activity, (ii) the hydrophobic patch, which may anchor the protein to the membrane, (iii) the C-terminal end, that binds FNR via a repeat structure, and (iv) a central motif, which mediates the interaction with the Tic complex.

Tic62 Is a Bona Fide Dehydrogenase—Based on structural similarities, Balsera *et al.* (12) placed Tic62 in the extended family of short chain dehydrogenases. Now we were able to

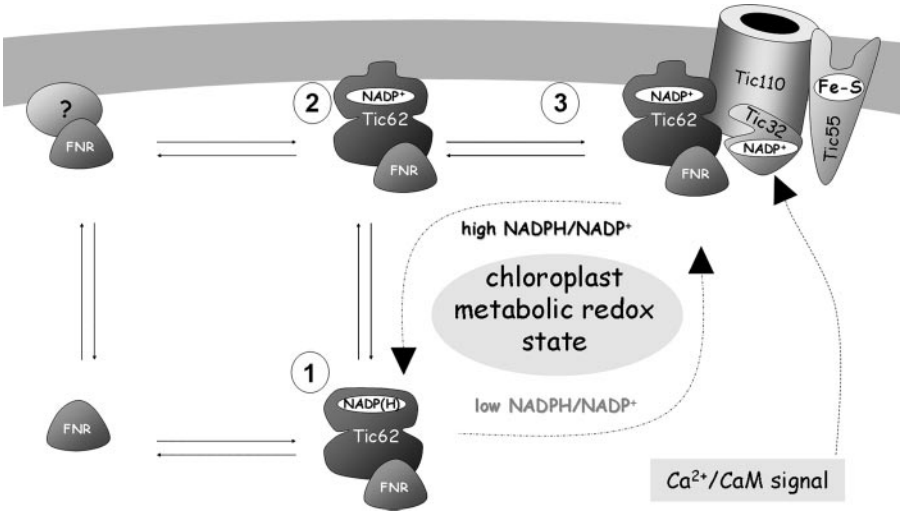

FIGURE 8. **Schematic model of the events taking place during the Tic62 shuttling.** In chloroplasts isolated in the light, a fraction of the Tic62 pool is found soluble in stroma, strongly associated to the FNR (1). If the redox conditions change to oxidized levels (low NADPH/$NADP^+$ ratio), Tic62 migrates to the envelope (2), supposedly supported by the formation of a hydrophobic patch on the surface of the N-terminal module. Ultimately, this change in localization seems to increase the affinity for the Tic complex (represented by Tic110 and the other two redox components Tic55 and Tic32), which is bound via the middle region of the Tic62 protein (3). High NADPH/$NADP^+$ conditions lead to an inverse movement: dissociation from the Tic complex (3-2) and increased solubility, including a stronger association with FNR (2-1). Additionally incorporated into the schematic drawing are the stromal and envelope-associated pools of FNR, which are not bound to Tic62, but may be involved in other complexes, as well as the Ca^{2+}/CaM signal mediated by the second Tic dehydrogenase Tic32.

demonstrate experimentally that Tic62 is indeed a *bona fide* dehydrogenase with strikingly similar properties to Tic32. Its enzymatic activity requires a lipid environment and specifically uses NADPH for the dehydrogenase reaction, which cannot be substituted for by NADH (Fig. 5*B*). Furthermore, the N terminus containing the NADP(H)-binding site is necessary and sufficient for dehydrogenase activity. The question remains why two dehydrogenases are present at the Tic complex. While Tic62 and Tic32 might function, together with Tic55, in one electron transfer chain at the inner envelope, their similar properties could also suggest that they rather act as single redox regulators, which integrate signals coming via the Ca^{2+}/calmodulin system to Tic32 or the metabolic redox status via FNR to Tic62, respectively.

CONCLUSION

We found Tic62 to be present in both the stroma and envelope of chloroplasts. The data presented here demonstrate that this distribution is not static but is clearly affected by the chloroplast redox pool ($NADP^+$/NADPH ratio). Furthermore, the results shown in Figs. 3*C* and 4 may indicate the presence of two subpools in the envelope membrane, one Tic-bound and another pool free from Tic but still attached to the membrane. We developed a working model in an attempt to accommodate the results of this study and to visualize the possible implications on the Tic62 shuttling behavior (Fig. 8): Oxidized conditions (low NADPH) promote membrane binding and, ultimately, increase Tic complex affinity. Reduced conditions on the other hand lead to dissociation from the Tic complex and into the stromal compartment.

It is widely accepted that all steps of protein synthesis, including transcription, mRNA stability, translation, and many post-translational processes are highly regulated (1, 22). Additional control mechanisms at the level of protein import therefore seem very likely. Regulation at the Toc complex was found to be mainly mediated by two prominent GTPases Toc34 and Toc159 (6). At the Tic complex, protein translocation was recently described to be regulated by calmodulin binding to Tic32 (10), and the existence of three putative redox-acting components at the Tic translocon raises the intriguing possibility of an electron transfer chain present at the inner envelope and therefore of redox regulation of protein import. Two independent studies (4, 11) have demonstrated that the import of certain preproteins indeed seems to be influenced by altered redox conditions in the chloroplasts. Furthermore, it was described that after high-light stress of *Arabidopsis* leaves, expression of Elip (early light-induced proteins) genes was induced, while protein amounts in the chloroplasts were reduced at the same time. This was interpreted as a possible post-translational effect, including the possibility of a change in import efficiency (26). Interestingly, a similar effect was observed when expression profiles and protein levels in FNR-L1 knock-out mutants in *Arabidopsis thaliana* were investigated (13). Also in that study, several subunits of thylakoid protein complexes were found in lower amounts in the mutants compared with the wild type, although no differences in the corresponding nuclear transcript levels could be detected.

In conclusion, there are various indications for a redox-dependent regulation of protein import into chloroplasts, and based on the findings presented here, Tic62 seems to be the best candidate known as yet to be involved in this process. Whether it directly alters the import efficiency of a special subset of precursor proteins at the Tic complex or acts rather indirectly by mediating a redox-derived signal to the inner envelope remains to be investigated.

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Chapter 3

Preprotein import into chloroplasts via the Toc and Tic complexes is regulated by redox signals in *Pisum sativum*. (2009) by **Stengel A**, Benz P, Buchanan B, Soll J and Bölter B. *Molecular Plant*, in press

Preprotein import into chloroplasts via the Toc and Tic complexes is regulated by redox signals in *Pisum sativum*

Running title: Redox regulation of protein import into chloroplasts

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The import of nuclear-encoded preproteins is necessary to maintain chloroplast function. The recognition and transfer of most precursor proteins across the chloroplast envelopes is facilitated by two membrane inserted protein complexes, the translocons of the chloroplast outer and inner envelope (Toc and Tic complexes, respectively). Several signals have been discussed to regulate the import of preproteins. In our study we were interested in redox-based import regulation mediated by two signals: regulation based on thiols and on the metabolic $NADP^+$/NADPH ratio. We sought to identify the proteins participating in the regulation of these transport pathways and wanted to characterize the preprotein subgroups whose import is redox-dependent. Our results provide evidence that the formation and reduction of disulfide bridges in the Toc receptors and Toc translocation channel has a strong influence on import yield of all tested preproteins that depend on the Toc complex for translocation. Furthermore, the metabolic $NADP^+$/NADPH ratio influences not only the composition of the Tic complex, but the import efficiency of most, but not all, preproteins tested. Thus, several Tic subcomplexes appear to participate in the translocation of different preprotein subgroups, and the redox-active components of these complexes likely play a role in regulating transport.

Chloroplasts are photosynthetic organelles characteristic of algae and land plants. They appear to have originated from an endosymbiotic event in which an ancestral photosynthetic cyanobacterium was engulfed by a mitochondriate eukaryotic host cell (Margulis 1970). During evolution, most of the endosymbiont's genes were transferred to the host nucleus (Leister 2005), so that the majority of chloroplast proteins is translated in the cytosol and posttranslationally targeted and imported into the organelle (Jarvis & Soll 2001, Schleiff & Soll 2004). The constant adaptation of chloroplasts to developmental or environmental changes requires a flexible organelle proteome that is regulated at several levels, *i.e.,* transcription, mRNA stability and translation, posttranslational modifications, targeting of precursor proteins, translocation across the double membrane and, finally, protein turnover inside the organelle (Kessler & Schnell 2006).

The import of preproteins into chloroplasts is mediated by two hetero-oligomeric protein complexes located in the inner and outer envelope membranes, respectively, designated Toc (translocon at the outer envelope of chloroplasts) and Tic (translocon at the inner envelope of chloroplasts). The Toc translocon consists of five subunits (Stengel *et al*. 2007, Jarvis 2008): Toc159, Toc34 and Toc64, which function as receptor components responsible for binding precursor proteins (Kessler *et al*. 1994, Qbadou *et al*. 2006); Toc75, which constitutes the translocation pore (Schnell *et al*. 1994) and forms the Toc core complex together with Toc159 and Toc34; and Toc12, which is located in the intermembrane space

where it seems to form a complex with Toc64 and a Tic component, Tic22 (Becker *et al*. 2004a). The Tic complex in the inner envelope is thought to consist of at least seven proteins (Stengel *et al*. 2007, Jarvis 2008, Benz *et al*. 2008): Tic110, Tic62, Tic55, Tic40, Tic32, Tic22 and Tic20. Tic110, the most abundant protein of the Tic complex, forms the central translocation channel (Heins *et al*. 2002, Balsera *et al*. 2008). Three other Tic components were proposed to act as potential regulatory redox-active subunits (the "redox regulon"): Tic62, Tic32 and Tic55. Tic55 represents a member of CAO/PAO-like oxygenases, containing a Rieske-type [2Fe-2S] cluster and an additional mononuclear iron binding site (Caliebe *et al*., 1997). Tic32 and Tic62 both belong to the family of short-chain dehydrogenases and possess dehydrogenase activity *in vitro* (Küchler *et al*. 2002, Hörmann *et al*. 2004, Chigri *et al*. 2006, Stengel *et al*. 2008). Tic32 was originally identified as an interaction partner of the N-terminal domain of Tic110 (Hörmann *et al*. 2004) and found to be the target of Ca^{2+}/calmodulin (CaM)-regulation of protein import (Chigri *et al*. 2006). Tic62 was found to bind FNR (ferredoxin-$NADP^{+}$-oxidoreductase) specifically, and to act as a shuttle between chloroplast membranes and the stroma in a redox-dependent manner (Küchler *et al*. 2002, Stengel *et al*. 2008).

The import of nuclear-encoded preproteins into chloroplasts using the Toc and Tic machineries is considered to be the general import pathway. A small number of proteins, however, enter the organelle via alternative import pathways: *e.g.*, several outer envelope proteins, including most Toc components, lack a cleavable transit peptide and seem to insert into the outer envelope membrane directly and spontaneously from the cytosolic side (Stengel *et al*. 2007, Jarvis 2008). There are also examples of inner envelope proteins without cleavable N-terminal targeting signals, such as the chloroplast envelope quinone oxidoreductase homologue (ceQORH) and Tic32. The import of both of these proteins was found to be independent of the Toc/Tic machineries (Miras *et al*. 2002, Nada & Soll 2004). An additional chloroplast-targeting pathway has been suggested by which certain proteins (*e.g.,* carbonic anhydrase 1, CAH1) first enter the endoplasmatic reticulum (ER) with an ER signal peptide and are subsequently transported via the Golgi apparatus to the chloroplast as vesicle cargo (Villarejo *et al*. 2005, Radhamony & Theg 2006). While only a few examples of alternative import pathways are known, it is conceivable that other proteins also import independently of the Toc/Tic complexes since many nuclear-encoded chloroplast proteins lacking a cleavable transit peptide have been identified in the chloroplast proteome (Kleffmann *et al*. 2004).

The need to adapt to change in developmental and environmental conditions suggests that protein import into chloroplasts is regulated. Moreover, it has been shown that regulation can take place at several levels, notably (i) the cytosol, (ii) Toc complex and (iii) Tic complex. Phosphorylation of the preprotein transit peptide in the cytosol was shown to influence translocation rate (Waegemann & Soll 1996, Martin *et al*. 2006). Regulation of protein import at the level of the Toc complex can be achieved by GTP/GDP binding and by phosphorylation of the receptor components Toc34 and Toc159 (Kessler & Schnell 2004). Binding of GTP increases the affinity of the Toc34 receptor for the transit peptide (Becker *et al*. 2004b), whereas phosphorylation of Toc34 causes inactivation of the receptor. Furthermore, protein import into the chloroplast may be influenced by the formation of disulfide bridges in the outer envelope translocation machinery (Friedman & Keegstra 1989, Pilon *et al*. 1992, Seedorf & Soll 1995). Oxidizing agents such as $CuCl_2$ strongly inhibit preprotein binding and import in contrast to reducing compounds (*e.g.* DTT), which increase import efficiency. The target proteins responsible for these effects have, however, not been identified, and the import of only a limited number of precursor proteins has so far been investigated under these conditions.

There are also indications of regulating protein import at the level of the Tic complex. It was proposed that import might be regulated by calcium signals, with calmodulin being the mediator and Tic32 the sensor protein (Chigri *et al*., 2006, Chigri *et al*., 2005). More recently, Tic55, which contains a conserved CXXC motif, was identified as a thioredoxin target, prompting the suggestion that redox could control protein import through the inner envelope (Bartsch *et al*. 2008). Regulation can

also be mediated by stromal or metabolically driven redox signals - *e.g.*, the $NADP^+$/NADPH ratio has been shown to have a clear influence on the composition of the Tic complex. Under reducing conditions, *e.g.*, low a $NADP^+$/NADPH ratio, certain members of the redox regulon (Tic62 and Tic32) detach from the Tic110 channel (Chigri *et al*. 2006, Stengel *et al*. 2008). A regulatory effect of the metabolic redox state on import has, however, not been reported.

In the present study, we have investigated two mechanisms for regulating redox-mediated import in chloroplasts of *Pisum sativum*. On the one hand, we took a closer look at thiol-mediated import regulation and the relevant translocation components and precursor proteins. On the other hand, we determined whether metabolic redox state, which was earlier found to affect translocon composition and dynamics, alters import efficiency. Our results demonstrate that the previously reported stimulation of import by thiol-reducing agents can be achieved by a number of compounds capable of reducing disulfide bridges of the receptor and channel components of the Toc translocon. Changes in the metabolic redox state (*i.e.,* the $NADP^+$/NADPH ratio) also regulate protein import via a mechanism likely linked to the redox regulon of the Tic complex. Our data indicate that the import of only a subgroup of precursor proteins is regulated by stromal redox state, suggesting the existence of several Tic subcomplexes or a highly dynamic Tic translocon composition. From parallel experiments with the moss *Physcomitrella patens* and the green alga *Chlamydomonas reinhardtii* as model systems, we concluded that regulation of the Toc complex by thiol-disulfide exchange occurs widely and probably developed relatively early in evolution. Regulation of import by the stromal $NADP^+$/NADPH ratio, by contrast, seems to have been acquired much later.

Results

Reducing agents increase protein import efficiency

It was demonstrated some years ago that preincubation of chloroplasts with DTT stimulates the import of preferredoxin (Pilon *et al*. 1992). The reversible formation of disulfide bridges was, therefore, invoked as a potential mode of import regulation (Friedman & Keegstra 1989, Seedorf & Soll 1995). While the effect is clear, the particular proteins subject to this type of regulation have not been identified. Moreover, it is not known whether this mechanism is general or specific.

In an attempt to analyze the nature of this effect in more detail, we initially determined whether other reducing agents stimulate import (Table 1). To this end, we tested agents that reduce disulfide bridges in addition to DTT - TCEP, GSH, β-ME - for an effect on import of preferredoxin 3 (pFd3). As seen in Fig. 1A, preincubation of chloroplasts with each of these reductants effected a clear stimulation of import. Using DTT, we next investigated whether the observed effect was due to exposure of chloroplasts or translation product to reductant and observed the stimulating effect only with the chloroplast preincubation (data not shown). DTT and TCEP were the most efficient agents tested, increasing import yield by ~5-fold vs. 3-4-fold with GSH and β-ME. Thus, while there were differences, *i.e.*, dithiols (DTT) were more effective than monothiols (GSH and β-ME), we concluded that all disulfide reductants increased import efficiency.

It is known that, unlike monothiols, the vicinal SH-groups of dithiols effectively reduce thioredoxin (Trx) - a regulatory protein able to reduce and thereby activate, among other enzymes, $NADP^+$-malate dehydrogenase (Scheibe *et al*. 1990, Miginiac-Maslow & Lancelin 2002), thereby influencing the equilibrium between $NADP^+$ and NADPH in the chloroplast stroma. Thus, under our conditions, DTT could potentially act in two ways: on the one hand, by reducing disulfides in the translocation machineries, and on the other, by influencing the metabolic $NADP^+$/NADPH ratio. While monothiols could act in the former capacity, only dithiols could promote $NADP^+$-malate dehydrogenase activation. The TCEP-effected increase in import yield is of interest in this regard, because, unlike the other reductants tested, it is unable to cross chloroplast membranes (Hsu *et al*. 2005, Motohashi & Hisabori 2006) - an observation confirmed in our study (see below). Hence, the observed effects on import seem not to depend on the ability of the active

agent to reach the inner envelope or enter the stroma. Rather the effect seems due to the reduction of protein disulfides of the outer envelope.

In a related experiment, we investigated whether the stimulation of import by thiols (in this case DTT) is observed only with some preproteins or whether it is a general phenomenon. As seen in Table 2 and Fig. 1B, most, but not all tested preproteins showed a similar stimulation of import. Of the 17 candidates tested, only Tic32 and 2-cysteine peroxiredoxin (2-CysPrx) were unaffected (Table 2, Fig. 1C). Tic32 is known to follow a route that differs from the general Tic/Toc import pathway (Nada & Soll 2004). 2-CysPrs also appears to fall into this category, although this needs further investigation. Taken together, the results suggest that, while vicinal thiols and TCEP are most effective, protein import is also enhanced by monothiols. Further, the import of most preproteins (that depend on the general import pathway for entering the chloroplast) is stimulated by thiols.

Most Toc components contain conserved cysteines and form potential intermolecular disulfide bridges

The stimulation in the import of most precursor proteins by reducing agents seen above and the earlier finding that the inhibition of import by oxidants can be reversed by DTT (Seedorf & Soll 1995) suggests a role for disulfide bridges in regulating this process. Further evidence for this conclusion is provided by analyzing the amino acid sequence of Toc components. Alignment of the Toc159, Toc75, Toc64 and Toc34 sequences from flowering plants (*Pisum sativum*, *Arabidopsis thaliana* and *Oryza sativa*) and a non-vascular plant (*Physcomitrella patens*) revealed conserved Cys residues in each protein. Five conserved Cys can be found in Toc75 (Fig. 2A), four in Toc64 and one in both Toc34 and Toc159. Restricting the Toc159 sequence alignment to flowering plants increases the number of conserved Cys to three.

As a follow up to the sequence alignments, we determined which proteins of the Toc complex have the potential to form disulfide bridges by using diagonal 2D redox SDS-PAGE gels. For this purpose, outer envelope vesicles were either oxidized with $CuCl_2$ or reduced with DTT before application to non-reducing SDS-PAGE in the first dimension, and then to a reducing gel in second dimension (Fig. 2B). With the DTT-treated samples, all proteins were reduced and thus ran in a diagonal line in both dimensions. By contrast, proteins forming disulfide bridges on treatment with $CuCl_2$ shifted and travelled either below the diagonal line (intermolecular disulfides) or to the right of the diagonal line (intramolecular disulfides). Several spots were detected below the diagonal line with this method and were identified by mass spectrometry. Analysis revealed the presence of nearly all Toc components (Toc159, Toc75, Toc64 and Toc34), which was additionally confirmed using antibodies generated against Toc75 and Toc34. As Toc159, Toc75 and Toc34 run in a vertical line, they may be localized in the same complex and form hetero-oligomers under oxidizing conditions. In contrast, Toc64 did not follow the vertical line and thus could either have originated from another complex or formed homo-oligomers. Only one other protein, ribulose-1,5-bisphosphate-carboxylase/-oxygenase (RuBisCO) was detected. The presence of RuBisCO in the outer envelope vesicles is likely due to well-documented contamination since this protein is highly abundant in the stroma and, so far, cannot be completely removed during envelope preparation. The gel analyses indicate that Toc proteins are able to form intermolecular disulfide bridges, rendering them capable of blocking the Toc translocon and impeding preprotein binding to the outer envelope. The redox state of the thiol groups in the Toc complex could affect the efficiency of protein import directly.

The $NADP^+$/NADPH ratio in the chloroplast can be altered *in vitro*

Our data indicate that the redox state of thiol groups in the Toc translocon has a clear influence on the import efficiency of most precursor proteins (Figs. 1,2). To pursue this point further, we analyzed the effect of the stromal metabolic redox state on protein import. Previous studies showed that the $NADP^+$/NADPH ratio influences the composition of the Tic complex (Chigri *et al*. 2006, Stengel *et al*. 2008), suggestive of regulation linked to changing redox conditions. We, therefore, determined whether stromal

redox flux alters import efficiency. To this end, we treated isolated chloroplasts with compounds that serve as substrates for enzyme reactions in the stroma (*e.g.,* members of the Calvin-Benson cycle) that are linked to the generation of $NADP^+$ or NADPH. The addition of appropriate metabolic intermediates should change the $NADP^+$/NADPH ratio of the chloroplast stroma (Table 1): 3-phosphoglycerate (3-PGA) and oxaloacetate (OAA) shift the pyridine nucleotide equilibrium to $NADP^+$, whereas dihydroxyacetonephosphate (DHAP) and malate lead to higher NADPH amount (Malkin & Nigoyi 2000, Dennis & Blakeley 2000). OAA and malate are linked to the enzyme $NADP^+$-malate dehydrogenase, which is strictly dependent on reduced thioredoxin and thus requires the addition of a dithiol reductant such as DTT. By contrast, although accelerated by DTT, the interconversion of PGA and DHAP can proceed independently of DTT. To test the effects of these reactions on import, we treated isolated chloroplasts with the respective metabolites and then isolated the stroma and measured the amount of $NADP^+$. We found that, while the addition of DTT alone gave no obvious change in the amount of $NADP^+$, both OAA + DTT and PGA led to an increase in stromal $NADP^+$ (Fig. 3). By contrast, addition of either malate + DTT or DHAP decreased the level of $NADP^+$. The effect on $NADP^+$ was more pronounced with OAA/malate (2.4-fold increase and 1.7-fold decrease, respectively) compared to the PGA/DHAP system (1.2-fold increase and 1.4-fold decrease, respectively). Overall, the results clearly show that the level of $NADP^+$ in the chloroplast stroma can be altered by added metabolites.

Change in the $NADP^+$/NADPH ratio influences import efficiency

As the selected metabolites changed the stromal $NADP^+$/NADPH ratio (Fig. 3), we determined whether this alteration affected protein import efficiency. For this purpose, pFd3 was imported into isolated chloroplasts following preincubation with different redox-active agents or wash buffer (control). Our data confirmed that DTT clearly stimulated import (Fig. 4A vs. Fig. 1). Treatment with PGA led to increased import efficiency as well, although the stimulating effect was less than observed with DTT (~2-fold vs. ~4-fold). OAA alone has no effect on import efficiency, however, preincubation with OAA in the presence of DTT gave a clear enhancement of import (~5-fold) - more than with DTT alone. A similar effect was observed with PGA + DTT, which, again, enhanced import efficiency more than DTT alone (~6-fold vs. ~4-fold). Interestingly, the observed increase with PGA (~2-fold) and DTT (~4-fold) seemed to be synergistic as combination of both additives gave a ~6-fold increase. On the contrary, since OAA alone had no effect, the change observed with OAA + DTT was not synergistic. In this case, both additives are necessary to stimulate import, likely due to activation of $NADP^+$-malate dehydrogenase as seen above. $NADP^+$ generation by converting OAA to malate can occur only with DTT. The addition of OAA + TCEP did not cause an increased import yield compared to TCEP alone, nicely confirming that TCEP is not able to cross the chloroplast membrane boundary under the conditions applied, and is thus not able to influence the stromal redox state (Fig. 4B). In contrast to the enhancement of the import efficiency by $NADP^+$-producing compounds, the addition of the NADPH-generating substrates, malate and DHAP, either with or without DTT, gave no increase in import efficiency. Moreover, import yield seemed to be slightly reduced with malate + DTT or DHAP + DTT compared to DTT alone, reflective of increased NADPH (Fig. 4A). Taken together, the results suggest that stromal metabolic redox state (*i.e.* the $NADP^+$/NADPH ratio) influences protein import: $NADP^+$-generating additives enhance import efficiency, whereas NADPH-generating counterparts have no effect or even slightly inhibit import.

Import increase by $NADP^+$ is preprotein-dependent

The import of a selected precursor protein, pFd3, was found to be regulated by the stromal $NADP^+$/NADPH ratio (Fig. 4A). To ascertain whether the observed stimulation by $NADP^+$-generating compounds is a general effect or whether it is restricted to a particular precursor subgroup, we determined whether the import of other preproteins was also influenced by a high $NADP^+$/NADPH ratio. To this end, we selected a spectrum of precursors ranging from proteins involved in redox-related reactions and thus possibly imported in a redox-dependent manner to others participating in redox-independent pathways (Table 2). The

proteins tested include members of the Calvin-Benson cycle (GAP-A, GAP-B, FBPase, SSU, PGK), proteins involved in the light reactions of photosynthesis (FNR-L1, FNR-L2, Fd1, Fd2, LHC-B1.3), key metabolic enzymes such as those of the pentose phosphate pathway (G6PDH, 6PGDH) and $NADP^+$-MDH, a protein involved in the homeostasis of nucleoside triphosphates (NDPK), a peroxiredoxin linked to stress response and antioxidant defence (2-CysPrx) and a component of the Tic complex (Tic32). Import was assayed for all proteins after preincubation of chloroplasts with or without PGA. PGA was chosen because, unlike OAA, it clearly stimulated import without DTT (Fig. 4A). Thus, a potential import increase should be detectable directly, independently of enhancement by DTT. Most tested precursor proteins showed increased import yield after PGA treatment similar to that observed initially with pFd3 (Fig. 5A). This protein subgroup includes the two FNR isoforms (L1 and L2), the ferredoxin isoforms (Fd1 and 2), LHC-B1.3, all metabolic enzymes tested (G6PDH, $NADP^+$-MDH, 6PGDH) and several Calvin-Benson cycle enzymes (GAP-A, GAP-B, SSU). Interestingly, while protein import was generally stimulated by PGA preincubation, certain representatives were not affected, including those related to redox (FBPase, 2-CysPrx, PGK) and those not related (Tic32, NDPK) (Fig. 5B). The results suggest the occurrence of either multiple Tic complexes composed of different subunits or multiple preprotein subgroups subject to different types of regulation.

Import regulation via the $NADP^+$/NADPH ratio seems to be an evolutionary new regulation pathway

The increase in import efficiency by DTT or PGA was so far observed with the flowering plant, *Pisum sativum*. To determine whether these effects apply to non-vascular plants or green algae as well, we performed import experiments with chloroplasts isolated from (i) the moss *Physcomitrella patens* - a non-vascular plant separated from flowering plants such as *Pisum sativum* or *Arabidopsis thaliana* by approximately 450 million years of evolution, and (ii) the green alga *Chlamydomonas reinhardtii*. It has been reported that most Toc and Tic components are conserved between *Physcomitrella, Chlamydomonas* and flowering plants, although certain differences were observed. For example, the full-length form of Tic62 is found only in flowering plants, non-vascular plants and green algae contain only a truncated version lacking the C-terminal domain that interacts with FNR (Kalanon & McFadden 2008, Balsera *et al*. 2007). On the other hand, the Cys residues of the Toc components are highly conserved in the moss, opening the door to regulation by thiol-disulfide exchange. In view of this structural difference we compared the three systems for regulation of protein import.

As seen in Fig. 6, although the effect was less pronounced than with *Pisum sativum* (1.7- and 1.2-fold vs. 4-fold enhancement), DTT clearly enhanced import of FNR-L1 with *Physcomitrella* (Fig. 6A) and *Chlamydomonas* (Fig 6B) chloroplasts when examining levels of the mature protein. By contrast, PGA had no significant effects in either the absence or presence of DTT. When viewing the abundance of the precursor, the results additionally show that the binding of preprotein by *Physcomitrella* chloroplasts is also stimulated by DTT. Thus, like pea, moss and green algae seem to regulate protein import via the Toc complex by thiol-disulfide exchange, suggesting the mechanism is relatively old in terms of evolution. By contrast, evidence for import regulation by change in the stromal metabolic $NADP^+$/NADPH ratio has so far been only obtained conclusively with flowering plants. It remains to be seen how the Tic complex is regulated by in mosses and green algae.

Discussion

Redox-regulation of protein import at the outer envelope: the formation of disulfide bridges in the Toc translocon.

Regulation of protein import into the chloroplast is necessary to maintain the proteome of the organelle in a dynamic and flexible state. In this study, we carried out a detailed investigation of redox-related regulation pathways linked to the Tic and Toc translocation machineries of the inner and outer chloroplast envelope, respectively. Our study demonstrated that different types of disulfide-reducing agents (DTT, a dithiol; β-ME and GSH, both monothiols; and TCEP, a

phosphoric acid ester lacking thiol groups) clearly increased preprotein import, suggesting the participation of regulatory disulfide bridges. Furthermore, participating disulfide groups resided in members of the translocation machinery rather than in the precursor proteins. This agrees with an earlier study in which the Cys in preferredoxin were modified with iodoacetamide, thereby preventing the formation of disulfide bridges (Pilon *et al*. 1992). The import of the modified preproteins was still enhanced by DTT, suggesting that the import machinery of the plastid is the site of regulation. Several lines of evidence pointed to the receptor and channel proteins of the Toc complex as major regulatory sites. First, import stimulation was also achieved with TCEP, a disulfide reductant that cannot cross chloroplast membranes and thus is only able to interact with proteins on the cytosolic side of the outer envelope. Second, import not only of selected precursors like preferredoxin (Pilon *et al.* (1992), but the import of all tested preproteins using the general import pathway was enhanced by reducing agents. The only exceptions were 2-CysPrx and Tic32, a protein previously found to use an alternate import route independent of the Toc and Tic complexes (Nada & Soll 2004). The evidence suggests that all preproteins imported via the Toc translocon are subject to redox regulation. Third, the one to five Cys conserved in most Toc components (Toc159, Toc34, Toc75 and Toc64) could potentially participate in regulation. The high degree of conservation of these Cys in flowering and non-vascular plants is consistent with a regulatory role. Finally, diagonal 2D Redox SDS-PAGE analysis of outer envelope vesicles treated with $CuCl_2$ revealed the presence of intermolecular disulfide bridges between most Toc components (Fig. 2B).

Regulation of protein import could be linked to several mechanisms resulting in activation by the formation of sulfhydryl groups. First, an oligomer consisting of Toc159, Toc34 and Toc75 joined by disulfide bonds could inhibit import by simply blocking the Toc75 translocation channel. A link between sulfhydryl status of the Toc receptors and targeted preprotein is supported by the earlier finding that $CuCl_2$ prevents the binding of the precursor to the chloroplast surface (Seedorf & Soll 1995). Reduction of the active disulfide groups could also lead to greater flexibility of the Toc receptors - a condition likely necessary for efficient preprotein binding and subsequent transfer of the precursor to the Toc75 channel protein. Such a flexibility mechanism is consistent with the proposal that Toc159 acts as the motor facilitating movement of preproteins through the translocation channel (Schleiff *et al*. 2003). Heterodimerisation of GTP-Toc34 and Toc159 is apparently required for GDP/GTP exchange in the primary preprotein receptor Toc34, thereby leading to formation of the GDP-Toc34 precursor complex which shows decreased affinity for the preprotein. Under these conditions, preprotein would more readily dissociate from the receptor, allowing efficient transfer to the motor Toc159 (Becker *et al*. 2004b, Schleiff *et al*. 2003). Based on these observations, it seems likely that the formation of disulfide bridges between the Toc receptors fixes a protein conformation that is incompatible with a functional receptor motor cycle. This mechanism could explain the decrease in import following treatment with oxidants described above. However, while present data clearly demonstrate regulation of protein import by oxidation/reduction of Toc protein thiol groups *in vitro*, the physiological relevance of this mechanism will require further investigation. In pursuing the problem it should be borne in mind that regulation could be linked to oxidants produced under changing environmental or developmental conditions. Moreover, cytosolic regulatory thiol proteins such as thioredoxins or glutaredoxins could be participants.

The stimulation of protein import by reductants was also observed with the moss *Physcomitrella patens* and the green algae *Chlamydomonas reinhardtii*. The Cys residues in the Toc components were found to be highly conserved between *Physcomitrella* and flowering plants (like *Pisum sativum*), which is in line with our experimental findings. The wide distribution of redox control of protein import at the outer envelope suggests its development relatively early in evolution.

Overall, our data suggest that the formation of intermolecular disulfide bonds between receptor and channel members of the Toc complex is responsible for the dependency of protein import on redox - with oxidation

blocking and reducing enhancing the process. Furthermore, this redox effect is not specific for selected preproteins, but seems to apply generally to precursor proteins imported by the Toc pathway.

Redox-regulation of protein import at the inner envelope: the role of the stromal $NADP^+$/NADPH ratio.

An early indication that protein import into chloroplasts is regulated by stromal redox-related events grew out of differential import observed with chloroplasts from light- and dark-grown maize plants (Hirohashi *et al*. 2001). The light/dark changes could influence the redox state of protein thiol groups or the stromal redox system (*e.g.,* the $NADP^+$/NADPH ratio) which seem to affect the import characteristics of the organelle. The $NADP^+$/NADPH ratio emerged as a mechanism of regulation at the inner envelope following its influence on defining the shuttling of Tic62 between the inner envelope and stroma (Stengel *et al*. 2008). Tic32 was also linked to this ratio after observing its dissociation from the Tic complex in the presence of reduced pyridine nucleotides (Chigri *et al*. 2006). Thus, it was concluded that the composition of the Tic complex seems to depend on the metabolic chloroplast redox state.

However, until now, there was no connection between this ratio and the import of precursor proteins. The present finding that a high $NADP^+$/NADPH ratio favors import efficiency provides a functional basis for this mechanism that seems relevant to certain preproteins. There appear to be two preprotein subgroups with respect to transport across the inner envelope, each transported by a different Tic subcomplex. Tic110 in association with stromal chaperones could represent the core translocon and provide the force to drive translocation across the inner envelope. Further, the redox regulon could associate reversibly with Tic110, thereby changing its translocation capacity (Fig. 7). Since Tic110 is a very abundant protein of the inner envelope, while the other Tic subunits are more minor, it is possible that only a fraction of the Tic110 core complexes are subject to redox regulation at any given time. This could explain the response of only some preproteins to changes in the metabolic chloroplast redox state, given that their import depends on the presence of redox-regulatory subunits. Further, regulation by the metabolic $NADP^+$/NADPH ratio could be complemented by a thiol-based system linking the activity of Tic110 to change in environmental conditions via thioredoxin (Balsera *et al.* 2008). It remains to be seen to what this latter mode of control extends to proteins of the chloroplast. A further issue awaiting clarification is a comparative list of proteins transported by Tic110 under the control of thiol redox status with those whose import is regulated by the metabolic $NADP^+$/NADPH ratio. It is also not clear to what extent the observed dynamics in Tic composition might influence these different regulation pathways. Non-photosynthetic "redox-independent" proteins like NDPK might be imported continuously in an unregulated manner, and thus require only a core Tic translocon consisting of the Tic110 channel associated with stromal chaperones or the co-chaperone Tic40. Metabolic "redox-dependent" preproteins, on the other hand, might be imported without enhancement by the redox regulon if the demand of these proteins is low. Upon certain stress conditions, or at times of active photosynthesis and thus a high demand of certain preproteins, the recruitment of the redox regulon would lead to an increased import of this protein subgroup. This model provides an explanation for the presence of some Calvin-Benson cycle enzymes (PGK and FBPase) in the group of proteins that does not respond to change in stromal redox state. An alternate explanation envisions that regulation of these proteins is mediated not by differential import, but at other levels such as transcription and translation or posttranslationally by modulation of the enzyme activity, *e.g.* by stromal thioredoxin as is well known for FBPase (Buchanan & Balmer 2005). Interestingly, PGK and FBPase catalyze reactions that are not directly linked to NADPH, as they mediate either in substrate phosphorylation (PGK) or dephosphorylation (FBPase). This is in contrast to other Calvin-Benson cycle enzymes tested - *i.e.*, GAP-A and GAP-B - that directly convert NADPH to $NADP^+$. This feature might also explain the independence of the import of PGK and FBPase on the stromal $NADP^+$/NADPH ratio. It remains to be seen whether the proteins imported without the influence of the stromal $NADP^+$/NADPH ratio possess similar motifs

that promote redox-independent import under certain conditions. So far, no obvious similarities were found between these preproteins.

Finally, it has to be noted that protein import with isolated *Physcomitrella* and *Chlamydomonas* chloroplasts seems to proceed independently of the $NADP^+$/NADPH ratio, as the observed slight increase in import yield was not found to be significant. Our results suggest that regulation by the $NADP^+$/NADPH ratio was probably acquired relatively recently in evolution. This view is supported by bioinformatic information suggesting that full-length Tic62, and perhaps Tic32 as well, were the last components added as the import machinery evolved (Kalanon & McFadden 2008). We hypothesize that the redox regulon in the Tic complex is responsible for mediating the import regulation by the $NADP^+$/NADPH ratio, as its existence seems to be required for this regulation pathway. However, import regulation pathways need further investigation in non-vascular plants and green algae.

To sum up, our findings provide evidence for two mechanisms functional in the regulation of protein import into chloroplasts (Fig. 7): a relatively old system in terms of evolution that relies on the formation and disruption of disulfide bridges of the Toc translocon of the outer membrane (Toc159, Toc34, Toc75 and Toc64). The other pathway, acquired more recently and affecting only a subgroup of preproteins is linked to the stromal metabolic $NADP^+$/NADPH ratio and is comprised of members of the Tic translocon of the inner membrane (the redox regulon, Tic32, Tic55 and Tic62). While details await further work, these sites of regulation appear to be complemented by a third dithiol-specific mechanism linking the Tic110 channel directly to regulation by redox, thereby enabling the chloroplast to respond to fluctuating environments (Balsera *et al.* 2008). Our understanding of these regulatory systems is in its infancy, and the overall picture is incomplete. Present evidence suggests that protein import is regulated by a complexity of elements that permit redox to act at multiple levels - from precursor recognition at the Toc complex, to translocation across the Tic translocon, to the adjustment of the protein complement enabling the chloroplast to meet the changing needs of the plant.

Materials and Methods:

Plant material and culture conditions

Pea plants (*Pisum sativum* var. Arvica) were grown under a 14 h light / 10 h dark regime at 20 °C / 15 °C. For chloroplast isolation, plants were harvested after 9–11 days.

Physcomitrella patens protonema cultures were grown for 7-9 days in a semi-continuous stirred tank glass bioreactor (Hohe & Reski 2002), equipped with a marine impeller running at 500 rpm. The cultures are aerated with 0.3 vvm [(aeration volume)/(medium volume)/min] air and grown at 25°C under a photoperiod regime of 16 h light / 8 h dark with light supplied at an intensity at the surface of the vessels of 120 µmol/m^2 per second.

Chlamydomonas reinhardtii strains were grown in Tris-acetate-phosphate (TAP) medium containing 1% sorbitol as described in Schwarz *et al.* 2007.

In vitro transcription and translation

The coding regions including the transit peptides of the analyzed preproteins (Table 2) were cloned into the vector pSP65 (Promega, Madison, USA) under the control of the SP6 promoter. Transcription of linearised plasmids was carried as previously described (Firlej-Kwoka *et al*. 2008) Translation was carried out using the Flexi Rabbit Reticulocyte Lysate System (Promega, Madison, USA) in the presence of [^{35}S]-methionine for radioactive labelling.

Chloroplast isolation and protein import

Chloroplasts were isolated from leaves of 9-11 days old pea seedlings (*Pisum sativum* var. Arvica) and purified through Percoll density gradients as previously described (Keegstra & Yousif 1986, Waegemann & Soll 1995). *Physcomitrella patens* chloroplasts were isolated according to (Hofmann & Theg 2003), and Chloroplasts from cell wall-deficient *Chlamydomonas reinhardtii* strains carrying the cw15 mutation were isolated as described previously (Schwarz *et al.* 2007, Zerges & Rochaix 1998). Chloroplasts equivalent to 10 or 20 µg chlorophyll (for pea or *Physcomitrella/Chlamydomonas* chloroplasts, respectively) were incubated with wash medium as control or with 2 mM (DTT, PGA,

DHAP, OAA, malate), 0.2 mM (TCEP) or 4 mM (β-ME, GSH) of the indicated compounds (Table 1) for 20 min at 25°C and washed once in wash medium (330 mM sorbitol, 50 mM Hepes/KOH pH 7.6, 3 mM $MgCl_2$). An import reaction was subsequently carried out in 100 µl import buffer (330 mM sorbitol, 50 mM Hepes/KOH pH 7.6, 3 mM $MgSO_4$, 10 mM Met, 10 mM Cys, 2% BSA (w/v)), containing 3 mM ATP and maximal 10% (v/v) [^{35}S]-labelled translation products. Import reactions were initiated by the addition of translation product to the import/chloroplast mix and carried out for 10 min at 25 °C. Reactions were terminated by separation of chloroplasts from the reaction mixture by centrifugation through a 40% (v/v) Percoll cushion. Chloroplasts were washed twice in wash medium (330 mM sorbitol, 50 mM HEPES/KOH pH 7.6, 0.5 mM $CaCl_2$) and treated with 100 µg/ml thermolysin for 20 min on ice. The reaction was stopped by the addition of 5 mm EDTA, sedimentation of the chloroplasts and resuspension in Laemmli buffer (50 mm Tris pH 6.8, 100 mM β-ME, 2% (w/v) SDS, 0.1% bromophenol blue (w/v), 10% glycerol (v/v)). Thermolysin digestion was omitted in case of the *Physcomitrella* imports. Import products were separated by SDS-PAGE and radiolabelled proteins were analyzed by a phosphorimager or by exposure on X-ray films.

Diagonal 2D Redox SDS-PAGE

Outer envelope (OE) vesicles (45 µg protein), isolated by sucrose density centrifugation (Waegemann & Soll 1995) were either reduced with 100 mM DTT or oxidized with 50 µM $CuCl_2$ at 4 °C for 60 min. After solubilization with 1% SDS for 30 min at RT, thiol-reshuffling was prevented by alkylation with 100 mM iodoacetamide at RT in the dark for 30 min. An appropriate volume of non-reducing SDS sample buffer was added and the protein samples were subjected to electrophoresis in the first dimension on a 12.5 % acrylamide separating gel. After separation, gel lines were excised and incubated in SDS running buffer containing 100 mM DTT for 15 min at RT, subsequently washed with SDS running buffer and incubated with 100 mM iodoacetamide in the same buffer. The gel strips were then horizontally applied to another 12.5 % acrylamide gel (second dimension), and gels were subsequently immuno-blotted with antibodies generated against Toc75 and Toc34 or stained with coomassie. Spots of interest were excised, subjected to in-gel trypsin digestion and analyzed by a combination of MALDI-TOF and LC/MS/MS analyses in the Protein Analysis Unit at the Adolf-Butenandt-Institute (Ludwig-Maximilians-Universität, Munich, Germany). The MASCOT program (Perkins *et al*. 1999) was used to analyse the mass spectrometry data.

Determination of $NADP^+$ and NADPH in chloroplasts

Isolated pea chloroplasts (~ 0.5 – 1 mg chlorophyll) were incubated with 2 mM of the indicated compounds for 20 min at 25°C, washed once in wash medium (330 mM sorbitol, 50 mM Hepes/KOH pH 7.6, 3 mM $MgCl_2$), and disrupted in 1 ml 5 mM Hepes/KOH pH 7.6 by incubation on ice for 20 min, followed by centrifugation for 10 min at 20.000 x g. The measurement of the $NADP^+$ amount in the supernatant (= stroma) using a spectrophotometer at 339 nm and analysis of the data was carried out as described (Klingenberg 1985).

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Figure Legends:

Figure 1: The import of most precursor proteins is increased by the addition of reducing agents
(A) Isolated pea chloroplasts, equivalent to 10 µg chlorophyll, were incubated with the indicated reducing compounds (2 mM DTT, 0.2 mM TCEP and 4 mM β-ME and GSH) or with wash buffer as control for 20 min at 25°C. After subsequent washing, import (10 min at 25 °C) was initiated by adding translation product (pFd3). The samples were subsequently treated with thermolysin to remove preprotein bound to the chloroplasts. Import products, including 10% of the translation product, were separated by SDS-PAGE and radiolabelled proteins were analyzed by a phosphorimager. Results for a typical pFd3 import assay and quantification, including standard error bars for three independent experiments, are shown. (B) Import results of several precursor proteins showing import stimulation following preincubation with or without 2 mM DTT. (C) Import results of precursor proteins that did not respond to DTT treatment. Radiolabelled proteins were analyzed by a phosphorimager or by exposure on X-ray films. (D) Quantification including standard error bars is shown for three to five independent import experiments of all tested preproteins. TL: translation product; p: precursor protein; m: mature protein

Figure 2: Toc components with conserved Cys can form intermolecular disulfide bridges
(A) Alignment of the Toc75 proteins from *Arabidopsis thaliana* (AtToc75-III), *Pisum sativum* (PsToc75), *Oryza sativa* (OsToc75) and *Physcomitrella patens* (PpToc75). The conserved Cys are highlighted with black boxes. (B) Diagonal 2D Redox SDS-PAGE of 45 µg outer envelope vesicles following incubation with either 100 mM DTT (reducing conditions) or 50 µM $CuCl_2$ (non-reducing conditions) before subjecting samples to electrophoresis on a 12.5% gel in the first dimension. After separation, gel spots were excised, the gel strips were horizontally applied to another 12.5 % acrylamide gel (reducing second dimension) and gels were stained with Coomassie blue. Proteins identified by mass-spectrometry are identified with an arrow. (C) Immunoblots with antibodies against Toc75 and Toc34 of diagonal 2D Redox SDS-PAGE of 20 µg outer envelope vesicles following incubation with either 100 mM DTT or 50 µM $CuCl_2$. The spots identified below the diagonal line correspond to Toc proteins that were oxidized upon $CuCl_2$ treatment and are marked with asterisks.

Figure 3: $NADP^+$ content of the stroma can be altered by adding DTT and metabolites to isolated chloroplasts
For measuring stromal $NADP^+$, isolated pea chloroplasts (~ 0.5 – 1 mg chlorophyll) were incubated with 2 mM of the indicated compound for 20 min at 25°C, washed once and disrupted in Hepes buffer. The stromal fractions were isolated by centrifugation (10 min at 20.000 g). $NADP^+$ was analyzed enzymatically by measuring change in absorbance at 339 nm after addition of glucose-6-phosphate dehydrogenase. $NADP^+$ present in the untreated control chloroplasts was arbitrarily set to 1 and the amount in the other samples was normalized to this value. Results show the mean value and standard error bars for three or five (for DTT/OAA/malate and PGA/DHAP, respectively) independent experiments.

Figure 4: Changes in the $NADP^+$/NADPH ratio influence pFd3 import efficiency
(A) Import experiments after preincubation with 2 mM of the indicated compounds were performed as in Fig. 1. A typical result selected from three independent experiments is shown for pFd3 including the quantification with standard error bars. (B) Import experiments with pFd3 as precursor were performed after preincubation of chloroplasts with 0.2 mM TCEP and/or 2 mM OAA. A typical result from three independent pFd3 imports is shown with quantification including standard error bars. TL: translation product; pFd3: Fd3 precursor protein; mFd3: Fd3 mature protein

Figure 5: Stimulation of import by generated $NADP^+$ is specific for a subgroup of preproteins
(A) Results of several precursor proteins that were imported more actively after preincubation with PGA. Experiments were performed as in Fig. 1. (B) NDPK, 2-CysPrx, FBPase, PGK and Tic32 show no differences in import efficiency on PGA-treatment. A typical result of the import with each preprotein is depicted. (C) Quantification including standard error bars is shown for three to five

independent import experiments of all tested preproteins. TL: translation product; p: precursor protein; m: mature protein

Figure 6: Import with *Physcomitrella and Chlamydomonas* chloroplasts is increased on incubation with reducing agents, but not with $NADP^+$-producing compounds
Isolated *Physcomitrella* (A) and *Chlamydomonas* (B) chloroplasts equivalent to 20 µg chlorophyll were incubated for 20 min at 25°C with 2 mM PGA, DTT, both agents (for *Physcomitrella*) or with wash buffer as control. Import (15 min at 25 °C) was started by the addition of translation product (pFNR-L1). Import products, including 10% of the translation product, were separated by SDS-PAGE and radiolabelled proteins were analyzed by exposure on X-ray films. One typical result of the import is depicted, as well as the quantification including standard error bars of three to six independent import experiments. TL: translation product; pFNR-L1: FNR-L1 precursor protein; mFNR-L1: FNR-L1 mature protein

Figure 7: Current view for role of redox in regulating protein import into chloroplasts
Redox-regulation of protein import at the level of the Toc complex is mediated by the formation or reduction of disulfide bridges either in the Toc channel Toc75 or in the Toc receptors Toc159, Toc34 and Toc64. Reduction of disulfide groups leading to an increase in import yield can be achieved by adding reducing agents (*e.g.*, DTT). By contrast, oxidation of thiol groups (depicted by -SH), *e.g.* by $CuCl_2$, inhibits preprotein import by either blocking the translocation channel Toc75 or by preventing precursor binding to the receptor. Intermolecular disulfide bridges are formed between the Toc proteins, possibly leading to formation of a heterotrimer consisting of Toc159, Toc34 and Toc75 (indicated by the close proximity of the components) and/or to homo-oligomerisation of Toc64 (indicated by the additional Toc64 molecule). All preproteins whose import depends on the Toc complex seem to be influenced by this thiol-mediated regulation. At the level of the Tic translocon, redox-regulation by the $NADP^+$/NADPH ratio takes place in the stroma: high levels of $NADP^+$ stimulate import, whereas high NADPH slightly decreases import efficiency. At least two components of the redox regulon were found to dissociate from the Tic complex at high NADPH levels (Tic62 and Tic32) (Stengel *et al.* 2008, Chigri *et al.* 2006). According to the current view, a shuttling of the entire redox regulon (consisting of Tic55, Tic32 and Tic62, along with ferredoxin $NADP^+$-oxidoreductase, FNR) is assumed to take place, although this has yet to be demonstrated for Tic55. Thus, recruitment of the redox regulon to the Tic110 "core" complex, consisting of at least Tic110 as central translocation channel and associated chaperones (indicated as import motor complex), leads to the stimulation of import. This redox-related import regulation seems to apply to most chloroplast preproteins with redox-associated functions. By contrast, a second subcomplex of Tic translocons is proposed for Tic110 as channel with the associated motor complex. This latter complex provides basic redox-independent import capacity for all proteins. Thus, the Tic redox regulon may sense photosynthesis-derived redox signals and modulate protein import according to the metabolic requirements of the organelle through its dynamic association with Tic110. Translocon subunits that seem not to be involved in redox-regulation were omitted for simplicity.

Table 1: Summary of redox-active agents tested in chloroplast import assays

Chemical	Abbreviation used	Description
Dithiothreitol	**DTT**	$C_4H_{10}O_2S_2$, **reducing agent**, breaks disulfide bonds, **dithiol**, is able to activate several stromal enzymes by thioredoxin reduction
β-Mercaptoethanol	**β-ME**	$HOCH_2CH_2SH$, **reducing agent**, breaks disulfide bonds, **monothiol**, can act as a biological antioxidant
Tris(2-carboxyethyl)-phosphine	**TCEP**	$C_9H_{15}O_6P$, **reducing agent**, breaks disulfide bonds, irreversible reductant, more powerful than DTT and β-ME, **can not cross the chloroplast membranes**
Glutathione (reduced)	**GSH**	$C_{10}H_{17}N_3O_6S$, tripeptide (γ-L-Glutamyl-L-cysteinylglycine), **reducing agent**, breaks disulfide bonds (GSH is converted to its oxidized form glutathione disulfide GSSG), **monothiol**
3-Phosphoglyceric acid	**PGA**	$C_3H_7O_7P$, calvin cycle compound, leads to production of $NADP^+$, 3-phosphoglyceric acid + ATP + NADPH ➔ glyceraldehyde 3-phosphate + **$NADP^+$** + ADP + Pi
Dihydroxyacetone phosphate	**DHAP**	$C_3H_7O_6P$, calvin cycle and pentose phosphate pathway compound, leads to production of NADPH, dihydroxyacetonephosphate + $NADP^+$ ➔ 1,3 bisphosphoglycerate + **NADPH**
Oxaloacetic acid	**OAA**	$C_4H_4O_5$, intermediate of the citric acid cycle and gluconeogenesis, leads to production of $NADP^+$, oxaloacetic acid + NADPH ➔ malate + **$NADP^+$** (catalysed by malate dehydrogenase)
Malic acid	**malate**	$C_4H_6O_5$, intermediate in the citric acid cycle and calvin cycle, leads to production of NADPH, malate + $NADP^+$ ➔ oxaloacetic acid + **NADPH** (catalysed by malate dehydrogenase)

Table 2: Summary and description of precursor proteins analyzed

Name of precursor protein	Abbreviation used	Accession number	Description	Import influenced by DTT	Import influenced by $NADP^+$/NADPH ratio
Ribulose-1,5-bisphosphate-carboxylase/-oxygenase small subunit	SSU	tobacco/ soybean	**Calvin-Benson cycle enzyme**, small subunit of RuBisCO, mediates the first major step of carbon fixation, catalyzes either the carboxylation or oxygenation of ribulose-1,5-bisphosphate, in calvin cycle: generates 3-phosphoglyceric acid, **reductive** pathway, subject to redox modulation (thioredoxin-mediated activation)	Yes	Yes
Phosphoglycerate kinase	PGK	At3g12780	**Calvin-Benson cycle enzyme**, catalyzes phosphorylation of 3-phosphoglyceric acid to 1,3-bisphosphoglyceric acid, **reductive** pathway, subject to redox modulation (thioredoxin-mediated activation)	Yes	No
Glyceraldehyde 3-phosphate dehydrogenase A	GAP-A	At3g26650	**Calvin-Benson cycle enzyme**, subunit of the photosynthetic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalyzes **reduction** of 1,3-bisphosphoglyceric acid to glyceraldehyde-3-phosphate, generates $NADP^+$, subject to redox modulation (thioredoxin-mediated activation)	Yes	Yes
Glyceraldehyde 3-phosphate dehydrogenase B	GAP-B	At1g42970	**Calvin-Benson cycle enzyme**, second subunit forming the photosynthetic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), subject to redox modulation (thioredoxin-mediated activation)	Yes	Yes
Fructose-1,6-bisphosphatase	FBPase	At3g54050	**Calvin-Benson cycle enzyme**, involved in the regeneration of ribulose-1,5-bisphosphate for CO_2 fixation, **reductive** pathway, subject to redox modulation (thioredoxin-mediated activation)	Yes	No
Malate dehydrogenase	MDH	At3g47520	**Key metabolic enzyme**, catalyzes the conversion of malate into oxaloacetate and *vice versa* (reversible reaction), subject to redox modulation (thioredoxin-mediated activation)	Yes	Yes
Glucose-6-phosphate dehydrogenase	G6PDH	At5g35790	**Key metabolic enzyme**, catalyzes the first step in the **oxidative** pentose pathway, generates NADPH, subject to redox modulation (thioredoxin-mediated inactivation by light)	Yes	Yes
6-Phospho-gluconate dehydrogenase	6PGDH	At1g17650	**Key metabolic enzyme**, involved in **oxidative** pentose phosphate pathway, catalyzes irreversible decarboxylation of 6-phosphogluconate to ribulose-5-phosphate, generates NADPH, subject to redox modulation (thioredoxin-mediated activation)	Yes	Yes
Light harvesting complex protein B1.3	LHC-B1.3	At1g29930	Involved in **light-reaction of photosynthesis**, subunit of light-harvesting complex II (LHCII), which absorbs light and transfers energy to the photosynthetic reaction center	Yes	Yes
Ferredoxin-$NADP^+$-oxido-reductase L1	FNR-L1	At5g66190	Involved in **light-reaction of photosynthesis,** leaf-type ferredoxin:NADP(H) oxidoreductase L1, catalyzes final step in electron transfer reactions of photosynthesis (electron transfer from ferredoxin to $NADP^+$ generating NADPH), forms a heterodimer with FNR-L2	Yes	Yes
Ferredoxin-$NADP^+$-oxido-reductase L2	FNR-L2	At1g20020	involved in **light-reaction of photosynthesis,** leaf-type ferredoxin:NADP(H) oxidoreductase L2	yes	Yes
Ferredoxin 1	Fd1	At1g10960	Photosynthetic ferredoxin, iron-sulfur protein, involved in **light-reaction of photosynthesis,** last electron acceptor, leads to NADPH production catalyzed by FNR	Yes	Yes
Ferredoxin 2	Fd2	At1g60950	Photosynthetic ferredoxin, iron-sulfur protein, involved in **light-reaction of photosynthesis,** last electron acceptor, leads to NADPH production catalyzed by FNR	Yes	Yes
Ferredoxin 3	Fd3	At2g27510	**Non-photosynthetic ferredoxin**, expressed predominantly in non-photosynthetic tissues, mis-sorted to the intermembrane space in the light, but is efficiently imported into the stroma in the dark	Yes	Yes
Tic subunit 32kDa	Tic32	AY488758.1	Part of the **Tic complex**, belongs to the „redox regulon“, short-chain dehydrogenase, contains NADP(H)-binding site, target of Ca^{2+}/calmodulin (CaM)-regulation of protein import, does not use general import pathway by Toc/Tic complexes	No	No
2-Cys peroxiredoxin	2-CysPrx	At5g06290	Reduces toxic peroxides to their corresponding alcohols, member of the **antioxidant defence** system of chloroplasts, functions in H_2O_2-mediated signal transduction, can be reduced by thioredoxin	No	No
Nucleoside diphosphate kinase	NDPK	At5g63310	**Redox-independent protein**, involved in the homeostasis of nucleoside triphosphates (NTPs), involved in embryo and seed development and in the response to blue light and high ionic strength	Yes	No

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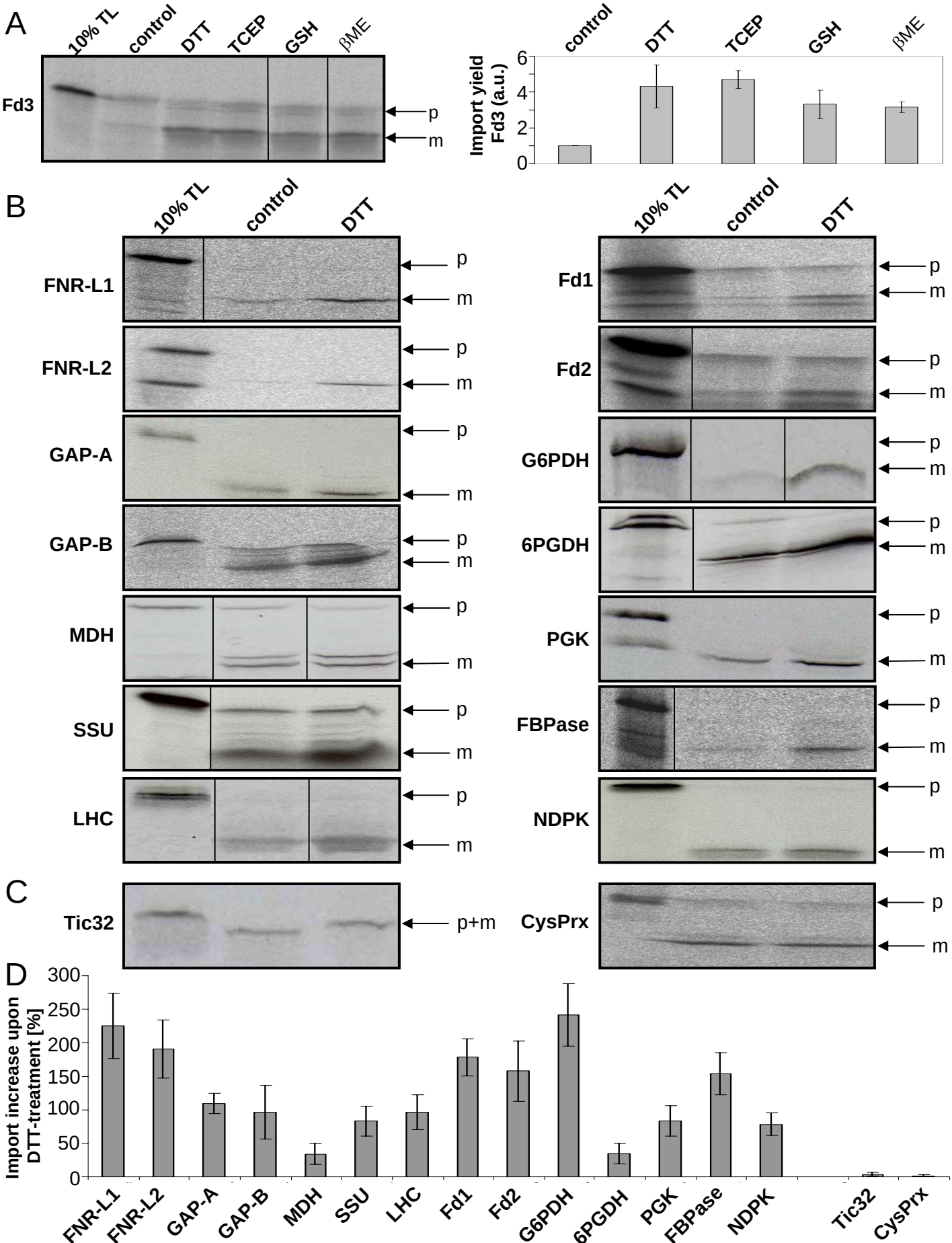
Figure 1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10% TL
control
DTT
TCEP
GSH
βME
Fd3
p
m
Import yield Fd3 (a.u.)
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2
4
6
B
FNR-L1
FNR-L2
GAP-A
GAP-B
MDH
SSU
LHC
Fd1
Fd2
G6PDH
6PGDH
PGK
FBPase
NDPK
C
Tic32
p+m
CysPrx
D
Import increase upon DTT-treatment [%]
0
50
100
150
200
250
300
FNR-L1
FNR-L2
GAP-A
GAP-B
MDH
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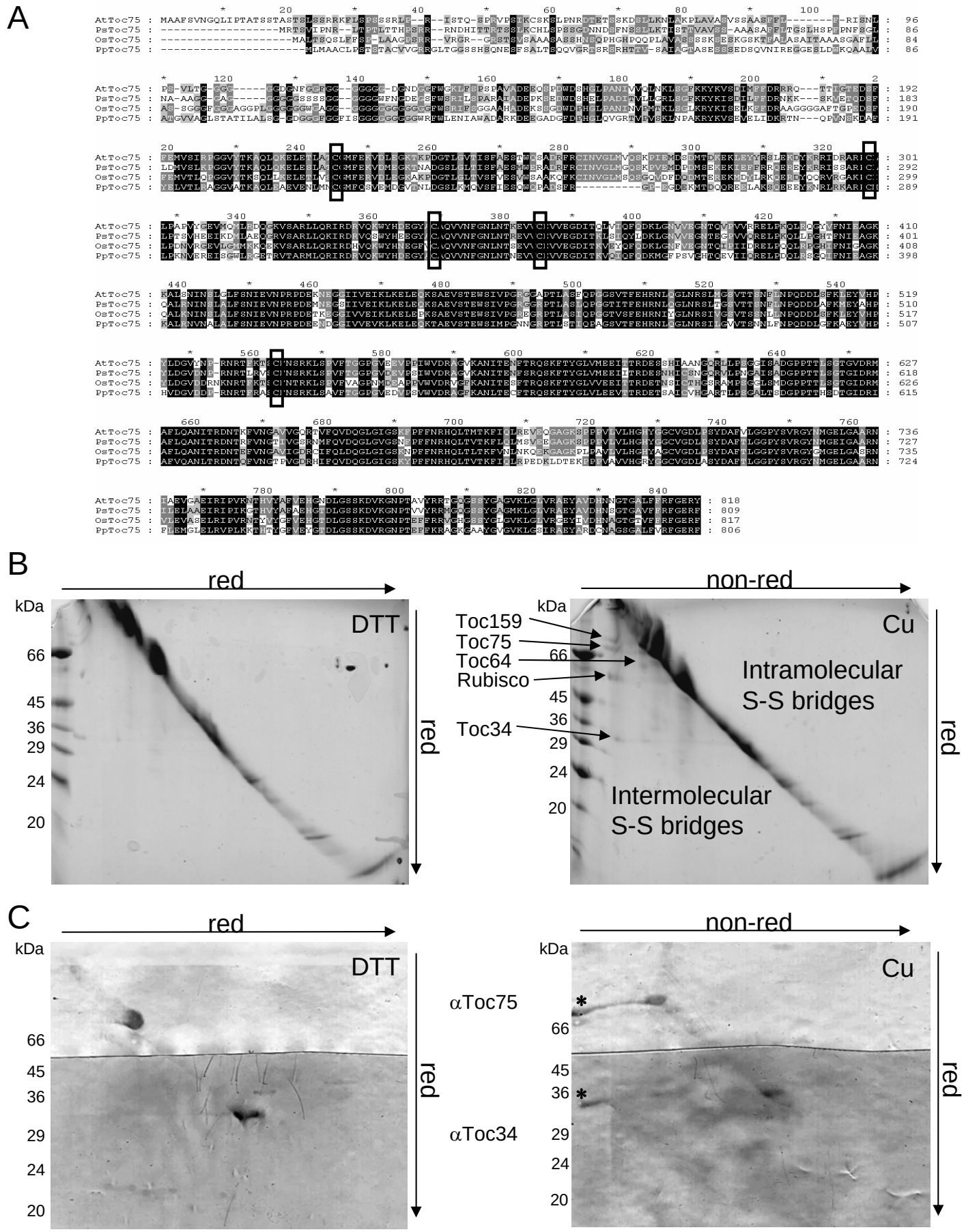
CysPrx

Figure 2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B
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Toc64
Rubisco
Toc34
Intramolecular
S-S bridges
Intermolecular
S-S bridges
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36
29
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αToc75
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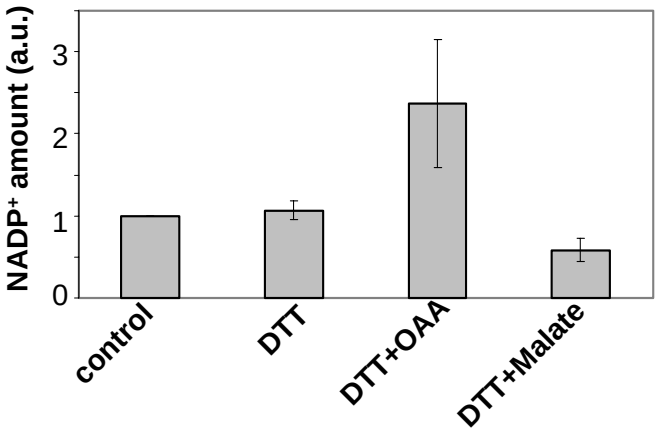
Figure 3



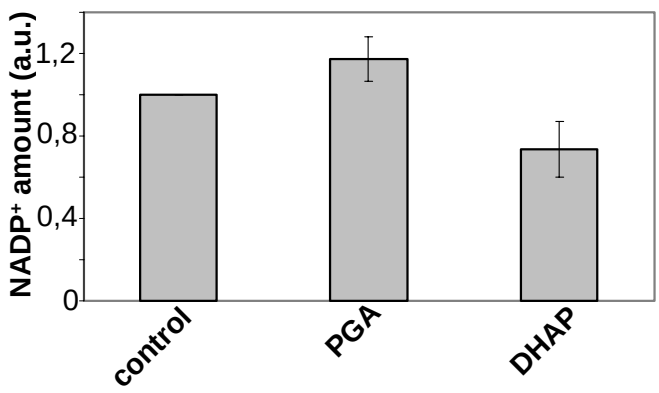
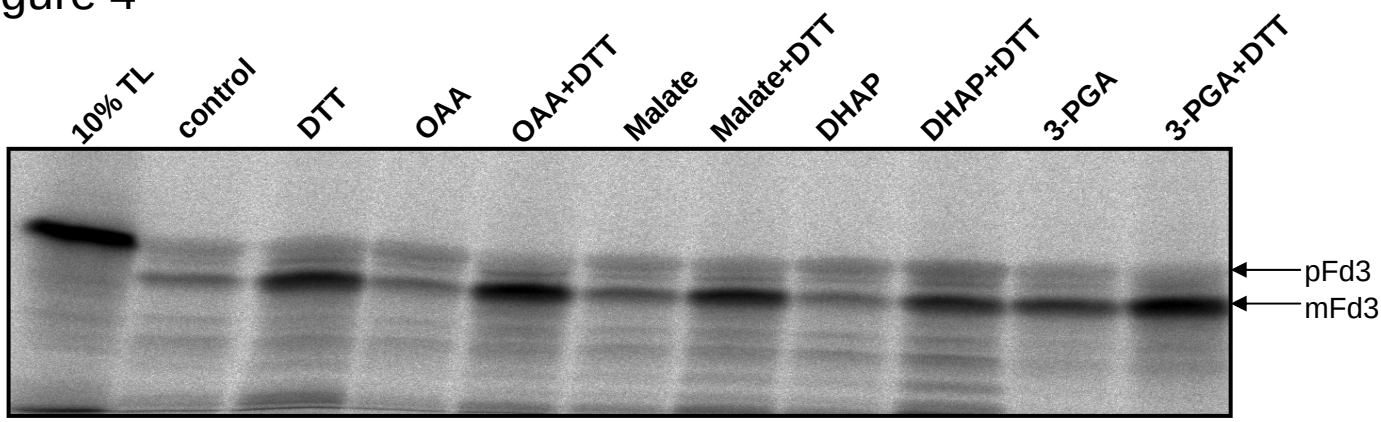
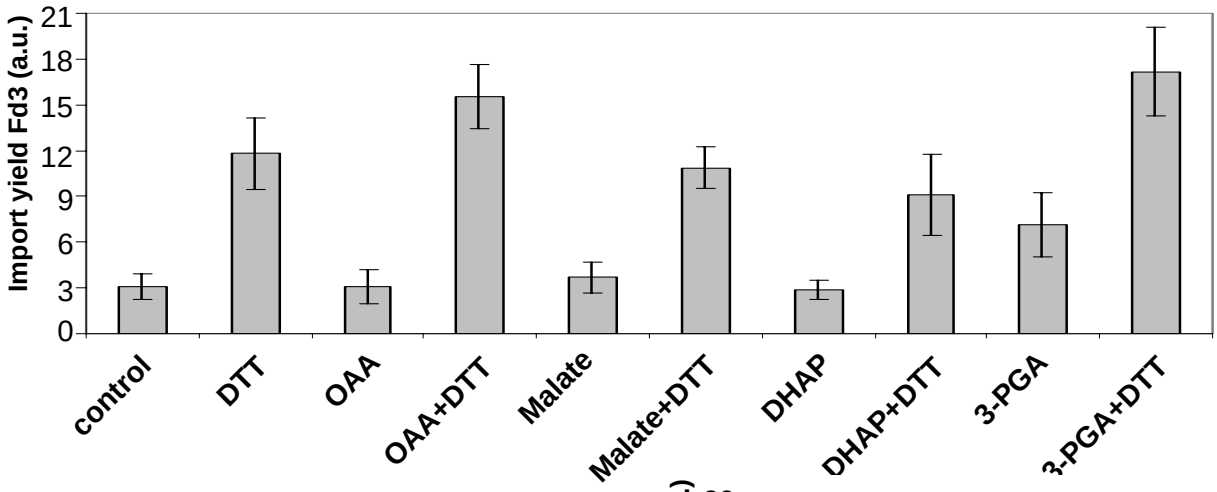


Figure 4

A





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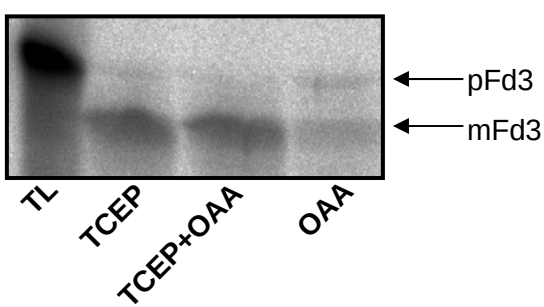


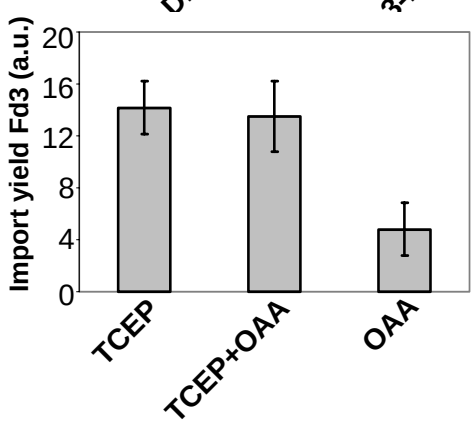
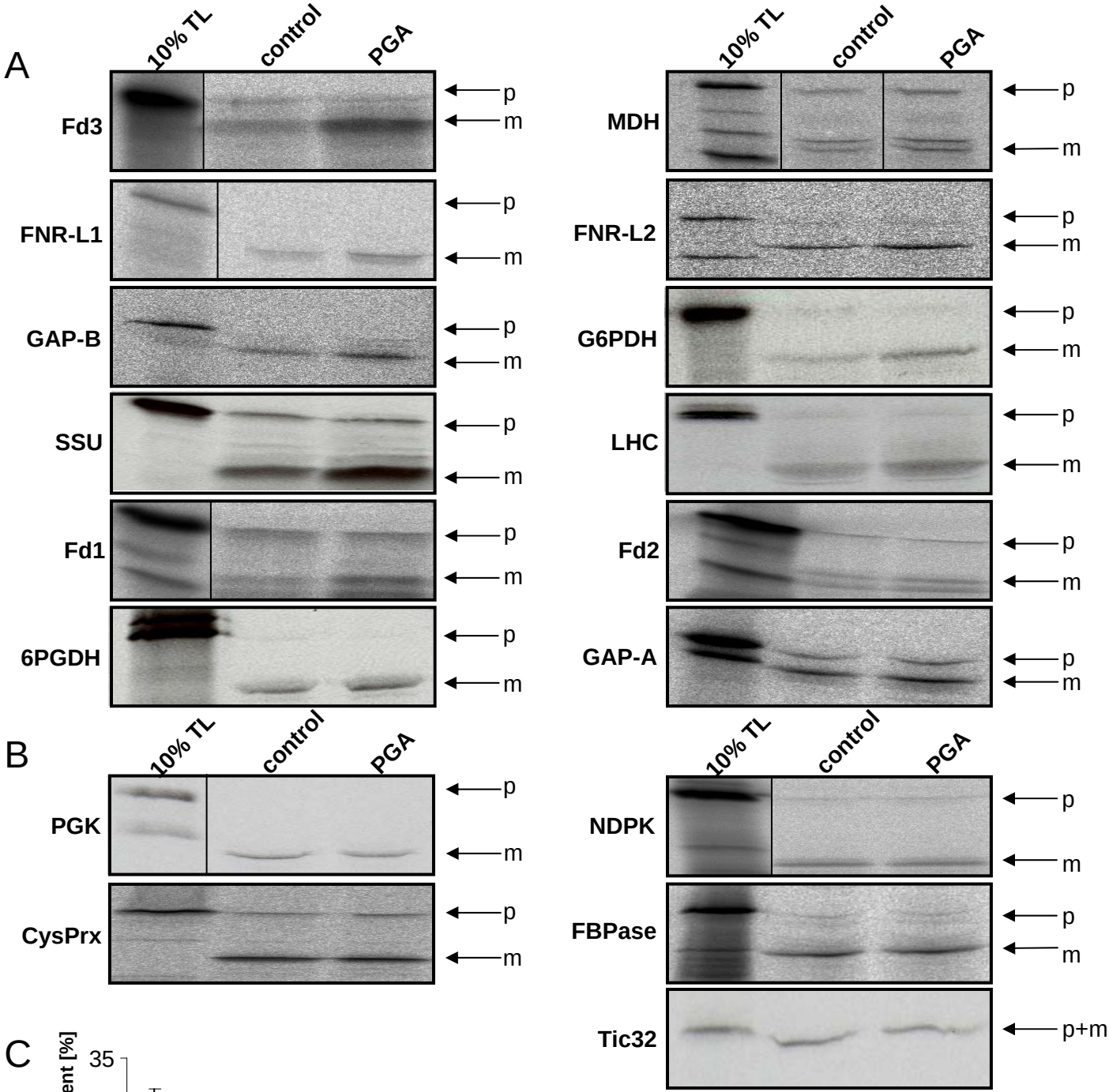


Figure 5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control
PGA
Fd3
FNR-L1
GAP-B
SSU
Fd1
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MDH
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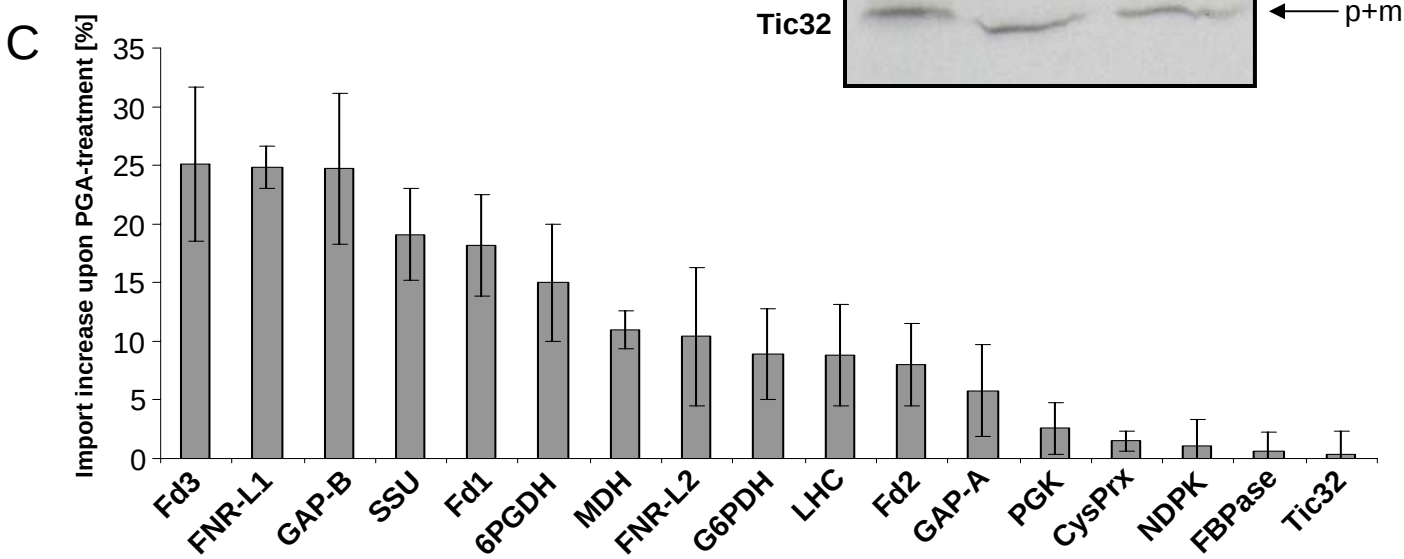
LHC
Fd2
GAP-A
p
m
B
PGK
CysPrx
NDPK
FBPase
Tic32
p+m


C
Import increase upon PGA-treatment [%]
0
5
10
15
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25
30
35
Fd3
FNR-L1
GAP-B
SSU
Fd1
6PGDH
MDH
FNR-L2
G6PDH
LHC
Fd2
GAP-A
PGK
CysPrx
NDPK
FBPase
Tic32

Figure 6

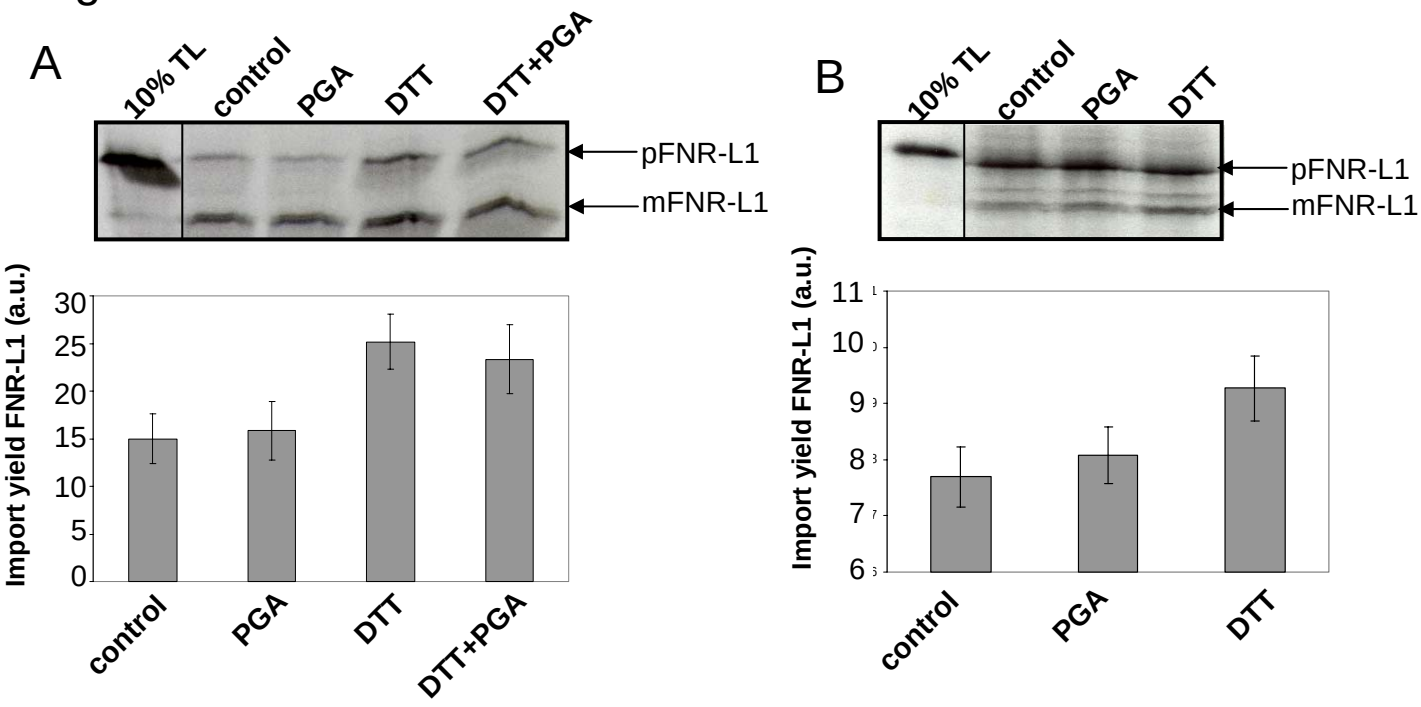
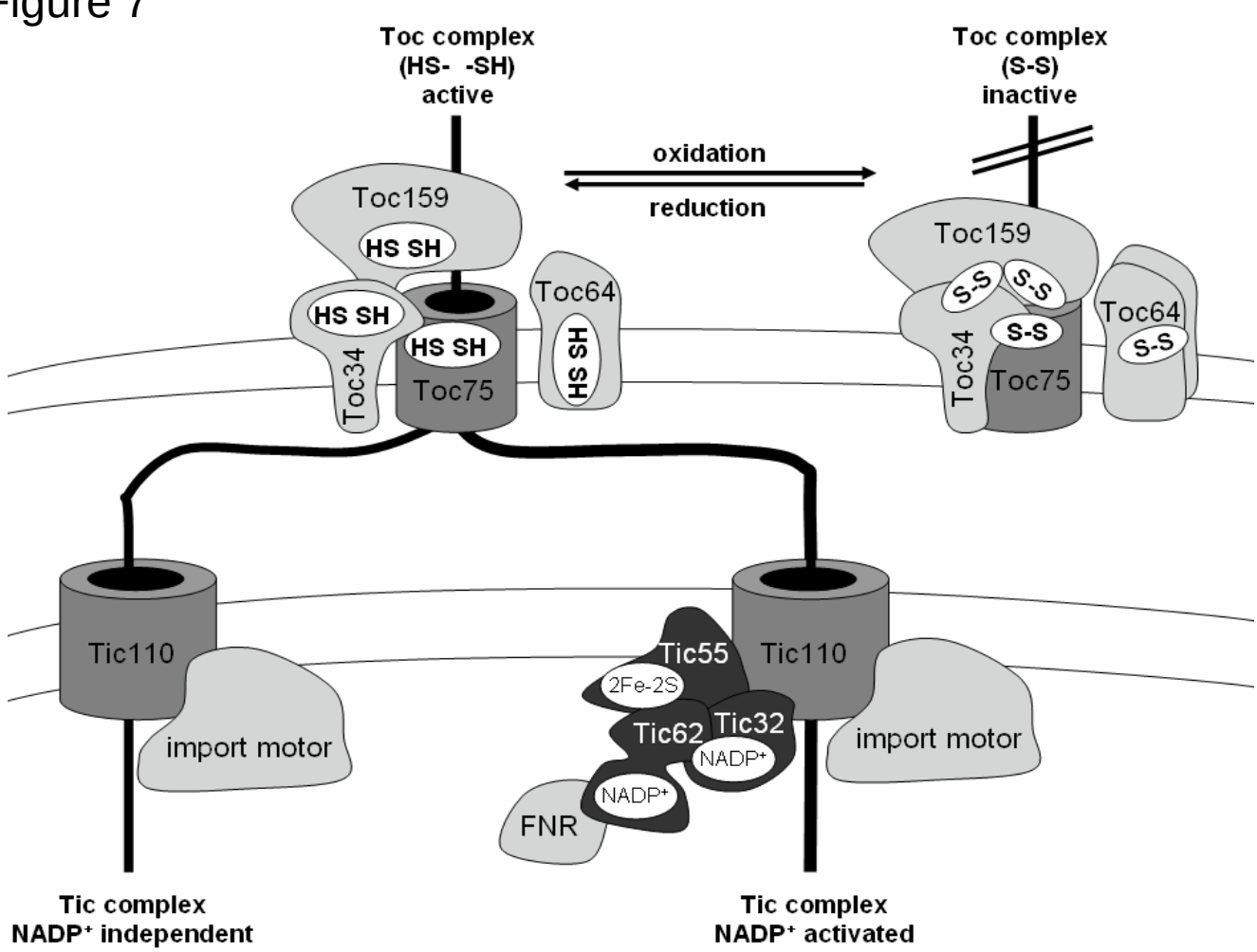


Figure 7



Discussion

Distinctive evolutionary features of Tic62

Tic62 was discovered as part of the Tic translocon in 2002 and was proposed as an interesting candidate for mediating redox regulation of protein import. It was found to bind pyridine nucleotides (in the N-terminal part) and to interact specifically with the photosynthetic enzyme FNR by a repetitive sequence located in the C-terminus (Küchler *et al.* 2002). So far, only the protein from *Pisum sativum* was investigated, and the present study expanded the information about Tic62 to all organisms that were found to contain at least one Tic62 homologue. Database searches performed revealed the existence of Tic62 in photosynthetic organisms only, including green plants, oxyphotobacteria and green sulfur bacteria (see Figures 3 and 4 in Chapter 1). Interestingly, full-length Tic62 proteins were only detected in flowering plant sequences (*e.g. Arabidopsis thaliana*, *Oryza sativa*, *Lycopersicon esculentum*). All other obtained Tic62-like sequences show similarities only to the N-terminal module, but completely lack the C-terminus including the FNR-interacting repeats. This suggests that the two domains of Tic62 are of different evolutionary origin.

The N-terminus seems to be rather old in terms of evolution, as it is highly conserved in all analyzed organisms and was even found in green sulfur bacteria (see Figure 2 in Chapter 1). These are very ancient anoxygenic phototrophic bacteria containing a type I (Fe-S) reaction center that use hydrogen sulphide (H_2S) as electron donor, in contrast to plants, where water is used for this purpose. Thus, not oxygen but sulfur is produced (Bryant & Friggard 2006). The fact that these bacteria also contain Tic62-like sequences is remarkable, as they are only distantly related to the oxyphototrophs, suggesting that the Tic62-related proteins are of very ancient origin. Furthermore, the function of the N-terminus seems to be important for the photosynthetic organisms since this part of the gene is conserved in all genomes. However, the exact function of the Tic62-like proteins remains speculative, as none of them has been studied in more detail, and the only feature they have in common is the NADPH-binding site.

In contrast to the N-terminus, only flowering plants contain the C-terminus with the FNR-interacting site (see Figure 1 in Chapter 1). The repetitive motifs consist of ~20 amino acids each that contain a highly conserved KPPSSPSP sequence flanked by conserved aromatic and charged amino acids. Since it is unlikely that all other organisms lost this domain, it can be speculated that the FNR-binding motif was acquired quite recently in evolution. Surprisingly,

no similarity to the C-terminus of Tic62 was found in any database analyzed, hence the origin of this polypeptide remains unclear. Other studies additionally indicated that Tic62 is an evolutionary young Tic component that became part of the Tic complex only recently in evolution (Kalanon & McFadden 2008, Reumann *et al.* 2005). It was thus hypothesized that the interaction with FNR was crucial for Tic62 to become a member of the Tic translocon. As the C-terminus with the Ser/Pro-rich FNR-binding repeats seems to be young in terms of evolution, it was surprising to see that this part is not strikingly homologue in flowering plants (see Figure 1 in Chapter 1 and Figure 5 in Chapter 2): whereas the repetitive motifs seem to be highly conserved, the remaining part of the C-terminal domains vary considerably in sequence and length. Moreover, even the number of repeats is not conserved: in *Pisum sativum* and *Oryza sativa*, three repeats can be found, in contrast to four in *Arabidopsis thaliana* and even seven in *Lycopersicon esculentum* (Figure 7). This suggests that only the repetitive motifs are responsible for FNR-interaction, independent of other C-terminal parts. Furthermore, although the binding to FNR is proposed to be important for the function of Tic62 in flowering plants, the C-terminus still seems to be the target of many evolutional changes, like mutations, deletions or duplications. It remains to be investigated if and how the number of repeats influences the binding of Tic62 to FNR.

Taking a closer look at the phylogenetic tree of Tic62, the analyses revealed that all the Tic62-related sequences can be divided in six groups (see Figure 3 in Chapter 1): Group I contains the full-length Tic62 sequences from flowering plants, although some truncated (N-terminal) Tic62-like proteins from non-vascular plants and green algae also belong to this group. All other sequences from groups II-VI are only homologous to the N-terminal part of Tic62, corroborating that only flowering plants possess a full-length Tic62 protein. However, even flowering plants additionally contain Tic62-like proteins belonging to groups II and V that are only homologue to the N-terminus. Sequences in group III are exclusively from cyanobacteria, and group VI only contains sequences from green sulfur bacteria (Figure 7). Another interesting aspect of the sequence analysis is obtained in proteins belonging to group V: while all other Tic62-related sequences from land plants were found to contain predicted TPs, this subgroup includes proteins without such chloroplast targeting signals. Thus, a localization outside the chloroplast can be proposed for the respective members of this group, although the lack of a predicted TP does not necessarily exclude the presence of the protein in the chloroplast (Nada & Soll 2004, Miras *et al.* 2002).

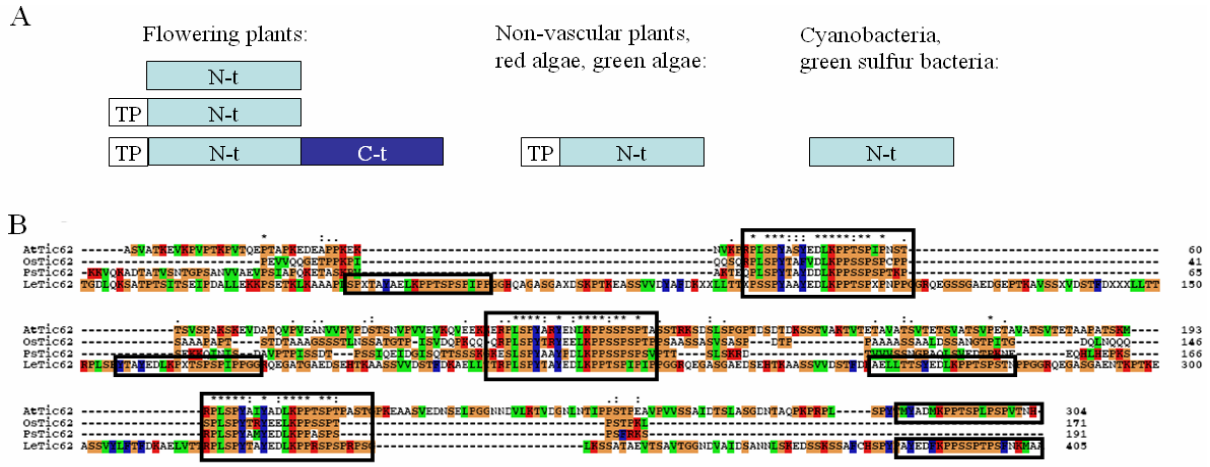


Figure 7: (A) Domain structure of Tic62 and Tic62-like proteins in flowering plants, non-vascular plants, cyanobacteria, algae and green sulfur bacteria. Only flowering plants contain full-length Tic62 consisting of the NADP(H)-binding N-terminus (N-t, light blue) and the FNR-interacting C-terminus (C-t, dark blue). Truncated forms (N-t only) are found as well. Most sequences contain an N-terminal TP (white) for chloroplast targeting, although sequences without a TP were also detected. Only homologues to the N-terminus were found in non-vascular plants, red algae, green algae (with TP), cyanobacteria and green sulfur bacteria (without TP). (B) Alignment of the C-terminus from flowering plants (*Arabidopsis thaliana*, *Oryza sativa*, *Pisum sativum*, *Lycopersicon esculentum*). The repetitive motifs responsible for FNR-binding are marked with black boxes.

Redox-dependent properties of Tic62

The redox-related features of Tic62 raised the question whether Tic62 itself is subject to redox regulation. Interestingly, a highly dynamic shuttling of Tic62 between the envelope and the stroma, dependent on the chloroplast redox state, was observed (see Figures 1 and 2 in Chapter 2). Furthermore, not only the distribution but also the interactions of Tic62 with the Tic complex and with FNR are redox regulated: oxidized conditions led to fast membrane binding and integration into the Tic complex. Reduced conditions on the other hand caused a transition to a soluble state in the stroma and an increased interaction with FNR (Figure 8). Based on the reversibility of membrane binding, a physiological role of the Tic62 shuttling was proposed. In that regard, it has to be noted that FNR was found to shuttle in a similar manner, as its amount in the membrane fraction was also increased in the presence of oxidizing agents (see Figure 1 in Chapter 2). Moreover, Tic32, another member of the Tic redox regulon, was described to be attached to the Tic complex only under oxidized conditions as well (Chigri *et al.* 2006), suggesting that the composition of the Tic complex is highly dynamic, and that it can be influenced by the metabolic $NADP^+$/NADPH ratio.

Structural analyses that were performed to investigate the nature of the Tic62 shuttling between the envelope and the stroma revealed that Tic62 does not possess any transmembrane helices that would anchor the protein very tightly into the membrane. However, a

hydrophobic patch could be located in the N-terminus that would allow a mere association of Tic62 with the membrane without completely spanning it (see Figure 5 in Chapter 1). This model offers an explanation for the observed shuttling behaviour and suggests that both the NADPH- and FNR-binding modules are exposed to the stroma. Moreover, a conformational change in Tic62 was detected upon binding of pyridine nucleotides: both the addition of $NADP^+$ and NADPH caused a change in the amount of α-helices and β-sheets, although it has to be noted that the two nucleotides led to different structural adaptations (see Figure 6 in Chapter 2). This might result in either shielding or exposure of the hydrophobic patch, which could explain the attachment to or dissociation from the membrane.

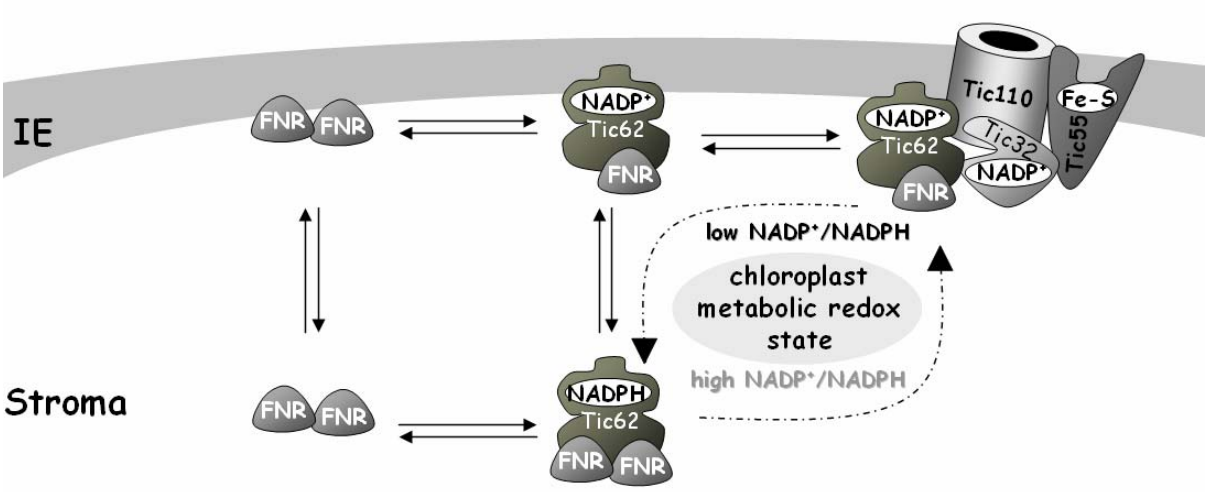


Figure 8 (adapted from Chapter 2, Figure 8): Redox-dependent shuttling of Tic62. The distribution and interactions of Tic62 are dependent on the metabolic redox state. Oxidized conditions (*i.e.* high $NADP^+$/NADPH ratio) lead to an enhanced binding of Tic62 to the membrane and to the Tic complex, whereas reduced conditions (*i.e.* low $NADP^+$/NADPH ratio) cause the detachment of Tic62 from the Tic complex and a solubilization into the stroma, where it interacts preferentially with FNR. The FNR was found to shuttle in a similar fashion.

Tic62 was proposed several years ago to bind pyridine nucleotides (Küchler *et al.* 2002), which was investigated closer in the present study. Structural predictions revealed a conserved Rossmann fold in the N-terminal domain (see Figure 5 in Chapter 1). Rossmann folds are structural motifs found in proteins that are able to bind to nucleotides such as $NADP^+$ or NADPH. They consist of three or more parallel β-strands linked by two α-helices (Rao & Rossmann 1973). The existence of such a characteristic structural module strongly suggested that Tic62 is indeed able to bind to pyridine nucleotides, which was confirmed experimentally later on: it was shown that Tic62 is a *bona fide* dehydrogenase that uses NADPH as electron donor and is able to transfer these electrons to artificial electron acceptors *in vitro* (see Figure 5 in Chapter 2). Interestingly, the properties of Tic62 were found to be strikingly similar to another dehydrogenase in the Tic complex, Tic32 (Chigri *et al.* 2006): both proteins require a

lipid environment for dehydrogenase activity and use exclusively NADPH for the reaction. Additionally, the C-terminal part of Tic62 was found unnecessary for dehydrogenase activity, and the N-terminus is sufficient for this function (see Figure 5 in Chapter 2). Thus, it can be postulated that dehydrogenase activity is a common trait of all Tic62-like proteins, even if they only contain the N-terminal part. Since in particular the NADPH-binding site is highly conserved, it can be hypothesized that the ability of the Tic62-like proteins to transfer electrons is an important function in all oxyphototrophs.

In the following, it was possible to closer localise the binding site of Tic62 to the Tic complex. While the N-terminus alone is able to mediate this interaction, the C-terminal part cannot bind to the complex, suggesting that it is exclusively responsible for the interaction with FNR. Moreover, a ~15 kDa fragment located in the center of the protein and containing sequences from both the N- and C-terminus was found to be sufficient for interaction with the Tic complex, indicating that the binding site might be localized in this region (see Figure 7 in Chapter 2).

All these data suggest that Tic62 consists of two modules that differ significantly in structure, function and evolution. The N-terminus displays a well-defined secondary structure (containing ~28% α-helices and 21% β-sheets), possesses dehydrogenase activity and is highly conserved from green sulfur bacteria to flowering plants. In contrast, the C-terminus forms a random coil structure, interacts specifically with FNR and is exclusively found in flowering plants. This model can now be extended to accommodate four functional regions distributed over the two modules (Figure 9): (i) the dehydrogenase domain in the extreme N-terminus, (ii) the hydrophobic patch responsible for membrane attachment, consisting of amino acids spread over large parts of the N-terminal domain, (iii) the FNR-binding repeats located in the C-terminus, and (iv) a central motif that is sufficient for mediating the interaction with the Tic complex.

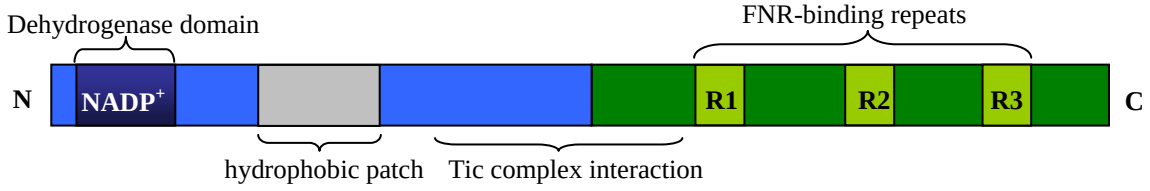


Figure 9: Model of full-length Tic62. Two structural, functional and evolutionary different modules, the N-terminus (blue) and the C-terminus (green) can be distinguished. Furthermore, Tic62 contains four functional motifs: the dehydrogenase domain (dark blue), a hydrophobic patch (grey), the Tic complex interaction site and the FNR-binding repeats (three in *Pisum sativum*, light green).

The results demonstrate that Tic62 is able to react very sensitively to redox changes in the chloroplast stroma and that it is able to adjust its localization and interaction affinities accordingly. Furthermore, interaction studies show that the association of Tic62 with FNR is surprisingly strong (see Figure 3 in Chapter 2). There is additional accumulating evidence for the importance of this interaction, as *e.g.* the amount of FNR is significantly reduced in ΔTic62 knockout plants and *vice versa* (P. Benz, A. Stengel, unpublished results).

The existence of two *bona fide* dehydrogenases (Tic62 and Tic32), a Rieske protein (Tic55) and the FNR with its FAD (flavin adenine dinucleotide) cofactor in one complex allows to speculate about a small electron transfer chain at the Tic translocon. Interestingly, dehydrogenases, flavin- and Fe-S-containing proteins have already been described as part of redox chains in the chloroplast envelopes (Jäger-Vottero *et al.* 1997). Although these spectroscopic measurements did not allow the identification of the participating proteins, it can be hypothesized that actually at least some members of the redox-active Tic components have been analyzed with this approach. The potential electron transfer chain could finally lead to changes in the import efficiency by modifying the channel properties of Tic110 in a yet unknown fashion. The sequence of the proteins involved in these hypothetical transfer reactions remains to be investigated, as well as the question whether all redox-active proteins participate in the same pathway. While Tic62, Tic32 and Tic55 might function in one electron transfer chain at the inner envelope, the similar properties of Tic62 and Tic32 could also suggest that they rather act in distinct redox regulation pathways. Thus, Tic32 might transmit signals coming from the Ca^{2+}/CaM system, while Tic62 forwards information about the metabolic redox status via FNR to the Tic complex. The strong interaction between FNR and Tic62 might explain the origin of the electrons involved in this process, as it is possible that one function of this interaction is a recruitment of FNR from the stroma or even thylakoids to the Tic complex in order to become part of the hypothetical electron transfer chain. Thus, FNR might be the "starting point" of these reactions, donating electrons to Tic62 which possibly mediates the subsequent electron transfer to Tic55 or Tic32.

Chloroplast protein import is regulated by various redox signals

Since it is clear that the $NADP^+$/NADPH ratio has an effect on the distribution of Tic62 and on the composition of the Tic complex, it was interesting to investigate a potential role of the metabolic redox state on protein import. The biochemical needs of the chloroplast differ markedly under varying conditions, *e.g.* active photosynthetic reactions might require higher

amounts of certain proteins at a given time, whereas less photosynthetic proteins could be needed *e.g.* during the night. The necessary regulation could take place at several stages, including protein import, which is clearly a bottleneck for the chloroplast protein content. There have already been some indications that the stromal redox system (*e.g.* the $NADP^+$/NADPH ratio) indeed affects the import characteristics of the organelle (Hirohashi *et al.* 2001), however, information about the nature of preproteins whose import is dependent on the stromal redox state as well as about the proteins that mediate this interaction was rather limited.

The results of the present study suggest that translocation of a variety of preproteins is dependent on the stromal redox state *in organello*: a high $NADP^+$/NADPH ratio favours import, in contrast to a low $NADP^+$/NADPH ratio, which has a slight inhibitory effect (see Figures 4 and 5 in Chapter 3). The observation that only a subgroup of mainly redox-related preproteins changes its import behaviour according to the metabolic chloroplast redox state led to the proposal of the existence of several Tic subcomplexes (Figure 10). A population of Tic translocons might only consist of Tic110 as central translocation channel, probably associated with import motor components like the co-chaperone Tic40 and stromal chaperones such as Hsp93. This "basic" Tic complex might be necessary for the import of redox-unrelated proteins which import is not adjusted by $NADP^+$, as their demand in the chloroplast is independent of the stromal redox state. This subclass of Tic translocons could also mediate the import of redox-related proteins when the need of these proteins in the chloroplast is low. Upon certain conditions, a high demand of redox-related proteins might be required, which leads to the dynamic recruitment of the redox-regulatory Tic components to the "basic" Tic machinery, forming the second subclass of complexes. These Tic translocons could then be able to sense photosynthesis-derived redox signals and regulate the import according to the stromal redox state, which allows an adaptation and fine-tuning of protein content in the chloroplast according to the metabolic requirements of the organelle. The observed highly dynamic Tic complex composition supports this idea (see Figure 3 in Chapter 2; Chigri *et al.* 2006), and the hypothetical subclasses of Tic machineries might exist in parallel in the chloroplast.

Interestingly, the presence of different subcomplexes was also proposed for the Toc complex (for review see Jarvis 2008), where the *Arabidopsis* isoforms of Toc159 (atToc159, atToc132 and atToc120) and of Toc34 (atToc34 and atToc33) are thought to have different substrate

specificities. AtToc159 seems to associate preferentially with atToc33, to form a distinct Toc complex responsible for the import of highly abundant, photosynthetic preproteins. In contrast, atToc132 and atToc120 are thought to function in the import of non-photosynthetic preproteins together with atToc34. It has to be mentioned however, that the interaction of atToc159 with atToc33 and of atToc132/atToc120 with atToc34 seems to be preferential, but not exclusive. How these Toc subcomplexes might interact with the different proposed Tic translocons requires further investigation.

It also has to be noted that not all redox-related proteins were found to import in a $NADP^+$-regulated manner: the Calvin-Benson cycle proteins FBPase and phosphoglycerate kinase (PGK) imported independently of the $NADP^+$/NADPH ratio (see Figure 5 in Chapter 3). There are several possible explanations why these two proteins with clear redox-related functions import without the control of the stromal redox state: firstly, in contrast to other tested Calvin-Benson cycle enzymes, the reactions catalyzed by FBPase (a phosphatase) and PGK (a kinase) are not directly linked to the conversion of $NADP^+$ to NADPH or *vice versa*. Thus, the $NADP^+$/NADPH ratio might not be a suitable regulation mechanism. Secondly, as regulation of chloroplast enzymes takes also place on other levels, like transcription or translation, an additional import regulation might be dispensable for some proteins. Moreover, it was described that the activity of both FBPase and PGK is controlled by the Trx system (for review see Montrichard *et al.* 2008), suggesting that the enzyme activity and not the protein amount is the more likely subject of regulation. Finally, it is furthermore possible that the applied import conditions do not require the redox-related import of these proteins, but that a "basic" unregulated import is sufficient under the given conditions.

Since the import studies performed on the flowering plant *Pisum sativum* indicate an influence of the stromal $NADP^+$/NADPH ratio on the import efficiency of a subgroup of preproteins, the question arises whether this regulation pathway exists also in non-vascular plants or even green algae. Interestingly, import experiments with chloroplasts of the moss *Physcomitrella patens* and of the green algae *Chlamydomonas reinhardtii* revealed that this kind of import regulation is not present in these organisms (see Figure 6 in Chapter 3). It has been described that most of the Toc and Tic components are highly conserved between *Physcomitrella* and also *Chlamydomonas* and flowering plants, although some interesting differences were found, in particular concerning the redox-related Tic proteins (Kalanon & McFadden 2008): Tic62 only exists in a truncated form in *Physcomitrella* and

Chlamydomonas, lacking the FNR-interacting repeats exclusively found in flowering plants (see Figure 4 in Chapter 1). Because the FNR-binding was proposed to be crucial for the function of Tic62 in protein import, it seems unlikely that these Tic62-like proteins are actually components of the import machinery. Furthermore, seven putative Tic32 homologues were found in *Physcomitrella*, all of which contain the characteristic motifs of short-chain dehydrogenases, but without further striking similarity to psTic32 (Kalanon & McFadden 2008). This leads to the question if they represent true functional Tic32 homologues as real parts of the Tic translocon in *Physcomitrella*. Regarding the striking differences of Tic62 and Tic32 between flowering plants and *Physcomitrella/Chlamydomonas*, it seems likely that such a kind of redox regulon does not exist in non-vascular plants or green algae as it was described in flowering plants. Thus, the comparison of the import regulation pathways between these organisms could provide insight into the importance of the Tic redox regulon import regulation by the stromal $NADP^+$/NADPH ratio. As protein import into isolated *Physcomitrella* and *Chlamydomonas* chloroplasts revealed that the $NADP^+$-controlled import stimulation is not detectable in these systems, it can be hypothesized that regulation by the $NADP^+$/NADPH ratio has been acquired only recently in evolution. Additionally, it is proposed that the presence of the redox regulon at the Tic complex is responsible for the detection of the stromal metabolic redox state and mediates the $NADP^+$-controlled regulation of protein import.

Another redox-related pathway that was found to regulate protein import is mediated by the reduction of disulfide bridges in the channel and receptor subunits of the Toc translocon (see Figure 1 in Chapter 3). These results nicely fit to previous studies that already proposed the involvement of disulfide bridges in the translocation process. They are based on the observed import stimulation by the reducing agent DTT as well as an inhibition of preprotein binding and import by oxidizing compounds such as $CuCl_2$ (Pilon *et al.* 1992; Seedorf & Soll 1995). Since this inhibition was found to be reversible, it was proposed that thiol groups might play an essential role in this process. In the present study, it was possible to verify this import regulation via the formation/disruption of disulfide bridges by using a variety of chemicals that are all able to reduce disulfides. Each compound tested caused a clear stimulation of protein import rate (see Figure 1 in Chapter 3). Moreover it was possible to show that the Toc channel, Toc75, and the Toc receptor components Toc159, Toc34 and Toc64 possess the ability to form intermolecular disulfide bridges *in vitro* and are thus potential targets of redox regulation (see Figure 2 in Chapter 3). However, not all of these proteins seem to work

together in thiol-mediated regulation of protein import: the data suggest that Toc75, Toc34 and Toc159 are able to form a heterotrimer, whereas Toc64 probably forms homo-oligomers upon oxidation (Figure 10).

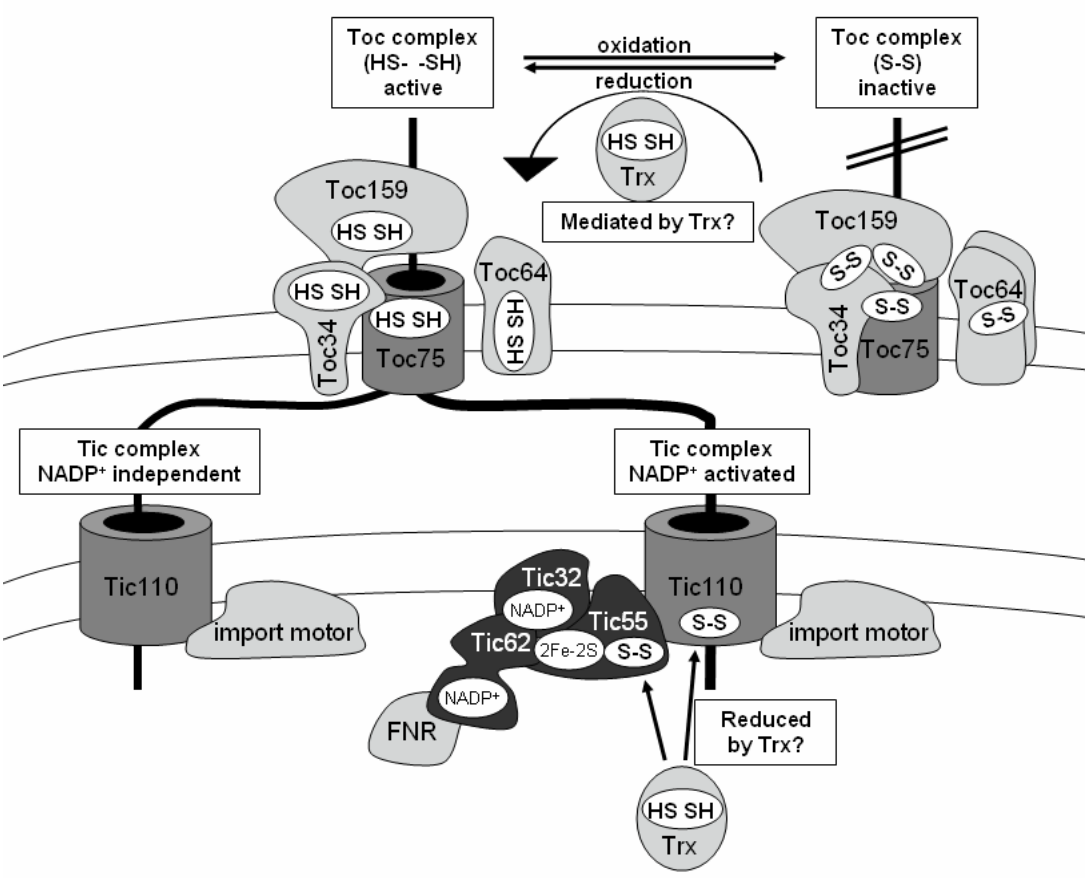


Figure 10 (adapted from Figure 7 in Chapter 3): Model of potential redox-related pathways involved in regulation of protein import. At the level of the Toc complex, redox regulation is mediated by the formation or reduction of disulfide bridges. Reduction (SH) leads to an increase in import yield, whereas oxidation (S-S) inhibits preprotein import, by leading either to formation of a Toc159/Toc34/Toc75 heterotrimer (indicated by the close proximity of the proteins) and/or to homo-oligomerisation of Toc64 (indicated by the additional Toc64 molecule). Reduction could possibly be mediated by cytosolic Trx. At the level of the Tic translocon, high levels of $NADP^+$ stimulate import, whereas high NADPH slightly decreases import efficiency and causes the dissociation of parts of the redox regulon (Tic62 and Tic32) from the Tic complex. Thus, recruitment of the redox regulon to the Tic110 "core" complex (Tic110 with associated chaperones, the "import motor") could lead to the stimulation of import, which seems to apply to most preproteins with redox-associated functions. By contrast, the Tic "core" complex provides basic redox-independent import for all proteins. Furthermore, Tic55 and Tic110 were found to be Trx-targets, which could provide an additionally redox-mediated import regulation. Translocon subunits that seem not to be involved in redox regulation were omitted for simplicity.

Two mechanisms of redox regulation by disulfide bridges are conceivable: firstly, the Toc159/Toc34/Toc75 oligomer that is observed after oxidation might block the translocation channel for incoming preproteins. Secondly, as all Toc receptors are targets of redox regulation it seems likely that already the binding of preproteins to the receptor components is affected. Thus, the reduction of disulfides, which was shown to have a stimulating effect on

protein import, might cause a higher flexibility and dynamics of the Toc receptors, necessary for efficient preprotein binding and subsequent handing over of the precursor to the channel protein Toc75. Disulfide bridges could "retain" the Toc components in an inactive conformation and prevent the hetero-oligomerisation of Toc34 and Toc159 required for efficient recognition of the preprotein and transfer from the primary receptor Toc34 to the motor protein Toc159 that subsequently pushes the preprotein through the Toc75 translocation channel (Becker *et al*. 2004a, Schleiff *et al.*, 2003). All these steps of precursor recognition and translocation are likely to depend on flexible protein structures.

With regard to the Toc translocon regulation, it was not possible to distinguish several precursor subgroups as it was the case for $NADP^+$-mediated redox regulation at the Tic complex. The only proteins that were found to import independently of the Toc oxidation status are precursors that use alternative import pathways and do not need the Toc translocon at all for entering the chloroplast (see Figure 1 in Chapter 3). Thus it can be concluded that the import of all preproteins that depend on the Toc complex for translocation is influenced by the reduction of disulfides in the Toc channel and receptors. Interestingly, import experiments that were again performed with chloroplasts isolated from *Physcomitrella* and of *Chlamydomonas* clearly demonstrated a similar increase in import yield upon reduction of disulfides in the Toc complex as observed in *Pisum sativum* (see Figure 6 in Chapter 3). The Toc core proteins (Toc159, Toc75 and Toc34) are highly conserved between *Physcomitrella*, *Chlamydomonas* and flowering plants, thus the wide distribution of this kind of redox regulation at the outer envelope suggests its development relatively early in evolution. Open questions are *e.g.* the environmental or developmental conditions under which this regulation pathway becomes important and how it might be regulated, *e.g.* by participation of cytosolic Trx. As the number of identified Trx targets increased remarkably during the last years (including Tic55 and Tic110, Bartsch *et al.* 2008 and Balsera *et al.* 2009), it seems almost likely that also protein import into the chloroplast might at least in part be influenced by this network. Thus, redox regulation mediated by Trxs could be an additional mechanism to control protein import both on the stromal and cytosolic side. It will be fascinating to investigate a potential influence of Trxs on both the Toc and the Tic complex in regard to protein import regulation in the future.

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List of abbreviations

Asc	ascorbic acid
at	*Arabidopsis thaliana*
CaM	calmodulin
CD	circular dichroism
C-terminus	carboxy terminus
CysPrx	cysteine peroxiredoxin
DHAP	dihydroxyacetonephosphate
DTT	dithiothreitol
FAD	flavin adenine dinucleotide
FBPase	fructose-1,6-bisphosphatase
Fd	ferredoxin
FNR	ferredoxin-$NADP^+$-oxidoreductase
FTR	ferredoxin–thioredoxin reductase
FTS	ferredoxin-thioredoxin system
GSH	reduced glutathion
GSSG	oxidized glutathion
HAR	hexammineruthenium trichloride
IE	inner envelope
IMS	intermembrane space
le	*Lycopersicon esculentum*
MDH	malate dehydrogenase
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NBT	nitroblue tetrazolium
NEM	N-ethylmaleimide
N-terminus	amino terminus
NTR	$NADP^+$–thioredoxin reductase
NTS	$NADP^+$-thioredoxin system
OAA	oxaloacetic acid
OE	outer envelope
PGA	3-phosphoglyceric acid
PGK	phosphoglycerate kinase

pp	*Physcomitrella patens*
Pro	proline
ps	*Pisum sativum*
Rubisco	ribulose-1,5-bisphosphate-carboxylase/-oxygenase
SDR	short-chain dehydrogenase
Ser	serine
SSU	ribulose-1,5-bisphosphate-carboxylase/-oxygenase small subunit
TCEP	tris(2-carboxyethyl)-phosphine
Tic	translocon at the inner envelope of chloroplasts
Toc	translocon at the outer envelope of chloroplasts
TP	transit peptide
TPR	tetratricopeptide repeat
Trx	thioredoxin
β-ME	β-mercaptoethanol

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Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die vorgelegte Dissertation von mir selbstständig und ohne erlaubte Beihilfe angefertigt wurde.

München, den

Anna Stengel

Erklärung

Hiermit erkläre ich

- dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.
- dass ich mich **nicht** anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

München, den

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