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-NAD⁺ 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, NO₂ 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) FdIII and the ferredoxin-NAD⁺- oxidoreductase isofom 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 Ca²⁺/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 ocytochrome P450-type oxygenases containing a Rieske-type [2Fe-2S] cluster and a di-iron (ferrodoxin) binding site, the second domain of Tic62 is a thioredoxin-like domain containing an NADP⁺/NADPH binding site.

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3 The abbreviations used are: Fd, ferredoxin; Asc, ascorbic acid; At, Arabidopsis thaliana; CD, circular dichroism; CP, chloroplast; FNR, ferredoxin-NAD⁺-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.
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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* without or with 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 \times g for 10 min at 4 °C, and the postribosomal supernatant was used for import experiments.

Chloroplastic 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-glucanate, 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 \times 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 \times g or for 2 h at 600,000 \times g, respectively. The pellet (membranes) and supernatant (stroma) were loaded completely and analyzed by SDS-PAGE and immunoblotting using antisera 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/Xhol, 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_{2}HPO_{4}/NaH_{2}PO_{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 PsTic62 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 H₂O, 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 NAPD(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 antisera 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 (Lα-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-methyleneanamide, 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 NADPH 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. 1A). 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. 1A). 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. 1A). 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 H₂O (data not shown). Although
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. 1A). 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. 1C). 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 H2O 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. 2A). 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 stroma and a total chloroplast membrane fraction in untreated control chloroplasts (Fig. 1A).

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. 2B). Moreover, even incubation of HAR-treated chloroplasts for 20 min in ambient light, which resulted in re-initiation 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. 2C). 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. 2D). 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.

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
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. 3A). 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. 3A, 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. 3A, compare lanes 3 and 6 of the control reaction). Similar results were obtained by co-immunoprecipitation experiments (Fig. 3B). 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. 3B). 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. 3C). This effect occurred 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. 3C).

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...
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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. 5A). 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. 5B, upper panel). Strikingly, dehydrogenase activity was only detectable after precubation 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. 5B, 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 LeTic62-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. 6A). In contrast, PsTic62-IA3 (C terminus of Tic62) revealed a disordered structure possibly involving poly (Pro) II helix features (Fig. 6B; 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. 6A). However, no obvious changes of the secondary structure could be observed upon

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

FIGURE 3. Oxidizing or reducing conditions influence the interactions of Tic62 with the Tic complex and FNR. A, 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 H2O (1 mm NADP+) or NADPH to the samples; proteins were pulled-down using Ni-beads, and the presence of FNR was investigated by immunoblotting. 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, NADPH for inner envelopes) compounds, solubilized with 1.5% decylmaltoside, and co-immunoprecipitated with αFNR. 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.
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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 Ni²⁺ 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 unveiled 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

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<sub>222</sub>), was altered by the presence of NADP⁺ (and less by NADPH; Θ<sub>222</sub>(Tic62-Nt) = −7951.51; Θ<sub>222</sub>(Tic62-Nt + NADP⁺) = −9019.75; Θ<sub>222</sub>(Tic62-Nt + NADPH) = −8157.50). This indicates a conformational change of Tic62 taking place upon binding to NADP(H) (Fig. 6C). Spectra analyses showed an increase of ~2–3% α-helices in the presence of NADP⁺ with a concomitant decrease of random coils (~2%).

**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 H₂O 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.

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remarkable relocation of Tic62 between the membrane fraction and the stroma (Fig. 1A). 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 H2O2 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 H2O2. 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 O2 at photosystem I, accelerating the production of O2/H2O2 (23, 24). The accumulation of H2O2 subsequently leads to oxidative stress and the inactivation of several important enzymes such as fructose-1,6-bisphosphatase, NADP-glyceraldehyde-phosphate dehydrogenase, ascorbate
peroxidase, and superoxide dismutase. These differing effects of HAR on the one and methyl viologen/H₂O₂ 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. 2D). 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. 2D), 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. 2B) 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. 3C). 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 vitro (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. 3A). 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 thylakoid-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. 5B). 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. 6A) 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. 6C), 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
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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 Ca²⁺/CaM signal mediated by the second Tic dehydrogenase Tic32.

Conclude

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 thali- ana 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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