The Transit Sequence of Ferredoxin Contains Different Domains for Translocation across the Outer and Inner Membrane of the Chloroplast Envelope*

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Deletion mutants in the transit sequence of preferredoxin were used in label transfer cross-linking assays to map the interactions of the transit sequence with the import machinery. The deletion mutants gave distinct cross-linking patterns to the Toc and Tic components of the import machinery, consistent with the binding and import properties obtained in in vitro import assays. The cross-linking results revealed two separate properties of the transit peptide: first the presentation of specific binding domains for the initial interaction with outer membrane components, and second the presence of different domains for interaction with the outer and inner membrane components of the transport machinery for full envelope translocation. The N-terminal Δ6–14 deletion blocked import of the precursor at the Toc components, whereas the more internal deletion Δ15–25 blocked import at the Tic components. The information for association with the outer and inner membrane components therefore resides in two separate but partly overlapping domains in the first 25 amino acids of the transit sequence.

One of the characteristic features of eukaryotic cells is their subcellular organization. Perhaps the most complex organization is found in higher plants. In addition to the nucleus, peroxisomes, mitochondria, and the secretory machinery, plant cells also contain plastids. The predominant plastid in leaves is the chloroplast. The majority of chloroplastic proteins are nuclear-encoded and are translated on cytoplasmic ribosomes. These proteins are post-translationally imported into the chloroplast, sorted within the chloroplast, and assembled into their functional native forms. Chloroplast biogenesis is therefore dependent upon a protein import apparatus that recognizes and translocates these proteins (for reviews see Refs. 1 and 2).

Recognition and translocation at the outer membrane are mediated by interactions of the transit sequence of the precursor with the Toc complex (translocon at the outer envelope membrane of chloroplasts) (3). The Toc complex consists of at least three proteins Toc34, Toc75, and Toc86 that mediate the initial interaction of the precursor with the chloroplast (4–6). Toc34 and Toc86 are GTP-binding proteins (7, 8) that interact directly with the precursor during recognition at the outer membrane (9). Recently it was shown that Toc86 is a proteolytic fragment of a larger 159-kDa protein, called Toc159 (10, 11). The 86-kDa fragment of Toc159 is sufficient for all stages of precursor interaction with the chloroplast in vitro (11) and Toc86 chloroplasts are fully import competent. The standard isolation protocol of chloroplasts, used in this study, results in the proteolysis of Toc159 into the 86-kDa fragment. Toc75 is a putative channel (5, 12, 13) and may participate in protein conductance at the outer membrane together with Toc86 by forming the receptor site of the import machinery (14).

Several components of the inner membrane translocon (Tics) have been identified: Tic20, Tic22, Tic55, and Tic110 (5, 15–18). Tic20 is a putative candidate for the inner membrane channel, whereas Tic110 may function as a stromal docking site for the hsp60 and ClpC molecular chaperone (19). The chaperones are proposed to participate in driving transport across the inner membrane or in folding of newly imported precursors (20). The stromal processing peptidase involved in processing newly imported preproteins also has been identified (21, 22).

For the unraveling of the molecular mechanism of translocation it will be essential to understand the interactions between the import machinery and the transit sequence of the precursor. Such an understanding requires that the information content and architecture of the topogenic information in the transit sequence is known. Transit sequences of different precursors are very heterogeneous in length and in secondary structure.

The precursor of the chloroplast stromal protein ferredoxin from Silene pratensis has been extensively characterized. In vitro this precursor is imported without the need for cytosolic factors, indicating that the precursor directly interacts with the chloroplast envelope (23). The transit sequence and mature part of the precursor are structurally independent (24). An extensive deletion mutant analysis of the transit sequence of ferredoxin in in vitro import, binding, and competition experiments with pea chloroplasts revealed different domains in the transit sequence (25). The first N-terminal domain mediates the initial binding; the second domain, the middle region of the transit sequence, is involved in envelope translocation after the initial recognition, and the third domain consisting of the C-terminal region is mainly involved in processing (25, 26). In this study examples of deletion mutants in each region were used that exhibited marked differences in their import characteristics. Deletion mutant Δ43–45 can be imported by the chloroplast with a reduced efficiency (~50% compared with wild type) but its binding is more severely affected. Processing is very inefficient in vitro. This mutant thus seems to be disturbed in the initial reaction, whereas translocation itself is less affected (25). In vitro deletion mutant Δ15–25 cannot be imported by the chloroplast but can bind to the chloroplast. It
is a strong competitor for import with the wild-type precursor (25). In contrast in vivo deletion mutant Δ15–25 can be imported (26). Two N-terminal deletion mutants were used. Deletion mutant Δ11–14 was selected as it can still be imported by the chloroplast although with a very low efficiency (~20% compared with the wild type). Deletion mutant Δ6–14 was selected because this deletion results in a defective transit sequence both in vitro and in vivo (25, 26). No deletion mutants were used from the region amino acids 26–42 as these can be imported with high efficiencies (25). All five deletion mutants can be processed in in vitro processing assays. To test the importance of the different domains of the preferredoxin transit sequence, we wished to investigate the interactions of these five mutant precursors with components of the import apparatus. Here, the deletion mutants were covalently cross-linked with the import machinery during binding and import using a label transfer cross-linking strategy.

The first domain, the N-terminal domain, was found to be necessary for outer membrane translocation, and the second domain, the middle region of the transit sequence, was required for inner membrane translocation, confirming the presence of functional domains within the transit sequence at the molecular level of the Toc and Tic components. The cross-linking data are consistent with the in vitro binding, import, and competition results, indicating that the competition of the deletion mutants with the wild-type transit sequence was indeed at the same components.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Plasmids pSPAF, pETFD-wt, pETFD-104, -371, -361, and -311 containing the full-length sequence of the wild-type ferredoxin from *S. pratensis*, a substitution serine at position 10 for a cysteine, and leucine 14 for a tryptophan or deletion in the transit sequence of amino acids (starting from the N terminus) 11–14, 15–25, and 43–45, respectively, were described previously (25, 27). Restriction enzymes, oligonucleotides, Percoll, Sepharose, the fast protein liquid chromatography system, and the Resource reversed phase chromatography column (3 ml) were from Amersham Pharmacia Biotech. Plasmid pET11-d was purchased from Novagen, U.K. The heterobifunctional cross-linking reagent **N**-[4-(2-azidosalicylamido)butyl]-3-[4-(2-pyridyldithio)propionamide] (APDP), was purchased from Pierce. Apyrase was purchased from Sigma. All other chemicals were of the highest grade available.

**Plasmid Constructions—**Standard procedures were used for restriction enzyme digests, analysis of restriction sites, ligations, and transformation of *Escherichia coli* (28). The three central cysteines of the *S. pratensis* wild-type sequence were exchanged for serine (position 88) and methionines (positions 93 and 96) in a first round of mutagenesis of pSPAF. In a second round of mutagenesis, the most C-terminal cysteine (position 126) was exchanged for a methionine. Mutagenesis was performed using the heteroduplex method (29). The BamHI-Nco fragment of pSPAF was then cloned in pET11-d resulting in pETFD-4, pETFD-403, -404, -471, -461, and -411. The mature sequences of the ferredoxin encoded by the last five plasmids contained only the most N-terminal cysteine. The sequence of the inserts in pET11-d was confirmed by dideoxy sequencing.

**Isolation of Preferredoxin—**Plasmids pETFD-wt, -372, -403, -404, -471, -461, and -411 were transformed into *E. coli* strain BL21 (DE3) (30). These strains were used to express the wild-type precursor, Δ6–14, wt-S103, wt-S104, Δ11–14, Δ15–25, and Δ43–45, respectively. Preferredoxin and the deletion mutants were purified essentially as described (27). Modification of Preferredoxin with [125I]APDP—The iodination of APDP was performed as described (14). The coupling to preferredoxin was performed essentially as described (14). During the procedure ~10% of the preferredoxin was not recovered over the G-25 spin columns.

**Chloroplast Isolation and Subfractionation—**Intact chloroplasts were isolated from 10–14-day-old seedlings by homogenization and Percoll silica gel gradient centrifugation as described previously (31).

To prepare chloroplast envelope membranes, intact chloroplasts were lysed under hypotonic conditions and separated into soluble and membrane fractions by differential centrifugation (32). The total membrane fraction was separated into envelope membranes and thylakoid membranes by floatation into linear sucrose gradients as described (5).

**Pressure Preconditioning, Impactor Binding, Impactor Release, and Binding Reactions—**For import and binding reactions, chloroplasts equivalent to 2 mg of chlorophyll were suspended in HS buffer (50 mM Hepes-KOH, pH 7.7, 0.33 M sorbitol), 2 mM ATP, and 2 mM MgCl2 in a total volume of 1 ml. For binding reactions to energy-depleted chloroplasts, chloroplasts were pretreated with 20 units of apyrase and 400 nm nigericin for 15 min at 26 °C. The protein was added to a concentration of ~200 μM in all experiments. For the import reaction, the chloroplasts were incubated for 20 min at 26 °C. Binding experiments were done under the same conditions except that the chloroplast was incubated for 30 min at 4 °C. The binding or import reactions were stopped by adding 1 ml of ice-cold HS buffer and intact chloroplasts were re-isolated over a 35% (v/v) Percoll in a HS buffer cushion. Chloroplasts were resuspended in 1 ml of ice-cold HS buffer, transferred to a glass Petri dish on ice, and irradiated from above with a Chromato-Vu transilluminator (UVP, Inc., Upland, CA) at 312 nm at a distance of 5 cm for 10 min with constant shaking. The chloroplasts were collected by centrifugation for 30 s at 6000 × g in a microcentrifuge and subfractionated as described above. Two thirds of the gradient containing ~90% cross-linked envelope precursor were collected, diluted with TE buffer (50 mM Tricine-KOH, pH 7.5, 2 mM EDTA), collected, and concentrated by centrifugation for 2 h at 40,000 × g. Equal amounts of protein/experiment as judged from the A280 were applied to SDS-PAGE.

**Sample Analysis—**All cross-linked samples were resolved by SDS-PAGE on 12% polyacrylamide gels. The radioactive signals were captured and quantified as described (14).

**Reversed Phase Chromatography—**For the import and binding reactions, chloroplasts equivalent to 2 mg of chlorophyll were resuspended in 1 ml of ice-cold HS buffer, transferred to a glass Petri dish on ice, and irradiated from above with a Chromato-Vu transilluminator (UVP, Inc., Upland, CA) at 312 nm at a distance of 5 cm for 10 min with constant shaking. The chloroplasts were collected by centrifugation for 30 s at 6000 × g in a microcentrifuge and subfractionated as described above. Two thirds of the gradient containing ~90% cross-linked envelope precursor were collected, diluted with TE buffer (50 mM Tricine-KOH, pH 7.5, 2 mM EDTA), collected, and concentrated by centrifugation for 2 h at 40,000 × g. Equal amounts of protein/experiment as judged from the A280 were applied to SDS-PAGE.

**Immunoprecipitation—**Immunoprecipitation of envelope components following membrane solubilization under denaturing conditions was performed with Toc34, Toc75, and Toc96 IgG coupled directly to Sepharose as described (14).

**RESULTS**

**Cross-linking Substrates—**The transit sequence of the *Silene* ferredoxin wild-type precursor, two full-length substitution mutants, wt-S104 and wt-S103, and four deletion mutants, Δ43–45, Δ15–25, Δ11–14 and Δ6–14, that are used in this study are outlined in Fig. 1A. These constructs were cloned in the pET11d-derived vector that allowed the synthesis of milligram quantities of precursor protein by isopropyl-1-thio-β-D-galactopyranoside induction of transformed *E. coli*. For use in the cross-linking study, all precursors were modified with the clevable, photoactivable reagent, [125I]APDP, by disulfide exchange at available cysteine residues. The position of the cysteines in the different substrates is outlined in Fig. 1B. The wild-type ferredoxin precursor contains five cysteine residues in the mature part of the precursor. To obtain specific cross-linking to the transit sequence, wt-S104, Δ11–14, and Δ15–25 were supplied with a cysteine in the transit sequence at position 10. In addition, the four most C-terminal cysteines in mutants wt-S103, wt-S104, Δ11–14, Δ15–25, and Δ43–45 were replaced with serine or methionine. To compensate for the low levels of binding observed with the Δ6–14 deletion mutant, all five cysteines were retained within its mature sequence to ensure sufficient cross-linking. The full-length ferredoxin wild-type was used as a control for this mutant substrate.

1. The abbreviations used are: wt, wild type; APDP, N-[4-(2-azidosalicylamido)butyl]-3-[4-(2-pyridyldithio)propionamide]; PAGE, polyacrylamide gel electrophoresis.

2. D. J. Schnell, unpublished observation.
After the coupling with \(^{125}\text{I}\)APDP, the radiolabel could be removed from the precursor by reducing agents, indicating that the cross-linker was indeed attached by disulfide linkages through cysteine residues (not shown). Modification with APDP was shown not to affect binding and import characteristics of a precursor protein (9, 14). Cross-linking of Precursors to Chloroplasts after Import—Chloroplasts were incubated with the ferredoxin precursors in the presence of 2 mM ATP to allow import for 20 min. After the import reaction the precursors were cross-linked to the envelope proteins. Because preferedoxin is fully import competent, no intermediates are expected after import. However, some of the deletion mutants might form intermediates under import conditions that are not readily detectable in standard import assays. The resulting cross-linking pattern would reflect the degree to which the mutants could engage the Toc and Tic machinery.

After the import reaction, the chloroplasts were irradiated with UV to induce photocross-linking. The chloroplasts were lysed and fractionated by sucrose density centrifugation into fractions corresponding to total envelope membranes. The envelope membranes were treated under reducing conditions, to cleave the cross-linker, and resolved by SDS-PAGE. Cleavage of the cross-linker with reducing agents releases the [\(^{125}\text{I}\)]APDP from the precursor but leaves it covalently attached to the proteins that were in close proximity of the precursor during the binding reaction. The cross-linked products were visualized directly by fluorography.

The results of the cross-linking are shown in Fig. 2. After import, cross-linking of the full-length wt-S104 precursor (Fig. 2, first lane) containing a cross-linker in the transit sequence resulted in one major cross-linked products of 86 kDa and minor cross-linked products of 75 and 55 kDa. Some precursor protein could also be detected because of intramolecular cross-linking. Cross-linking of the second full-length ferredoxin precursor, wt-S103 (Fig. 2, second lane), that did not contain a cross-linker in the transit sequence resulted in a similar cross-linking pattern. The cross-linking of the wild-type preferedoxin (Fig. 2, second panel, first lane) resulted in the same cross-linking pattern and also a 34-kDa band could be detected. This interaction may not be specific as this precursor contains five cysteines in the mature part of the precursor, which may be just in the vicinity of Toc34 as it inserts across the outer membrane. In similar cross-linking experiments the interaction was only seen in the absence of ATP (9).

To test whether the 34-, 75-, and 86-kDa bands were indeed Toc34, Toc75, and Toc86, an immunoprecipitation with anti-Toc34, -Toc75, and -Toc86 IgG coupled to Sepharose was performed (Fig. 3A). Solubilized cross-linked envelope proteins to the full-length wt-S104 precursor were subjected to sequential immunoaffinity chromatography on anti-Toc34, anti-Toc75, and anti-Toc86 IgG-Sepharose. The 34-, 75-, and 86-kDa bands were bound to their corresponding IgG columns identifying these bands as these envelope components. The identity of the 55-kDa protein is not known.

The cross-linking pattern of the most C-terminal deletion mutant Δ43–45 resembled the cross-linking pattern of the wt-S103 control substrate (Fig. 2, last lane). After import, Toc86 and Toc75 were the major cross-linked products, and the ratio Toc86/75 is similar to the wild-type control. Import of deletion mutants Δ11–14 and Δ15–25 resulted in the cross-linking of Toc86 and Toc75 in a ratio that is similar to the wild-type interactions. To identify the 14- and 21-kDa cross-linked products, envelope proteins from a similar import experiment as presented in Fig. 2 with deletion mutant Δ15–25 were subjected to reversed phase chromatography followed by SDS-PAGE and immunoblotting (Fig. 3B). The 21-kDa protein was recovered in the same fraction as Tic20 in previous experiments (15). This indicates that this band was most likely Tic20. The 14-kDa protein is most likely the same putative component of the import machinery as described before (9). The identity of the 55-kDa band remains unknown. The elution profiles of Toc34–75, and -86 were consistent with previously obtained data (15). Also on the Western blot probed with antibodies against Toc34–75, and -86, these labeled proteins could be identified as Toc34, -75, and -86 (not shown). Similar results were obtained with envelope proteins cross-linked to the full-length precursor wt-S104 (not shown). The identification of the 14- and 21-kDa proteins as Tic components indicates that during the import reaction with deletion mutants Δ11–14 and Δ15–25, the information for the correct interaction with the Tic components was lacking. In contrast the interactions with the Toc components were comparable to the interactions of the full-length transit sequence.
In cross-linking experiments with the most N-terminal deletion mutant Δ6–14 after import Toc86 could be detected as a minor cross-linked product, together with the 55-kDa band (Fig. 2, second panel, second lane). So the deletion of amino acids 6–14 resulted in a precursor that could only establish an interaction with Toc86.

Analysis of the Stroma after Chloroplast Import—To investigate which precursors indeed translocated into the stroma after import, a sample of the stromal fraction from the import experiments in Fig. 2 was analyzed. The results are shown in Fig. 4. The full-length precursors, wt-S103 and wt-S104, were detected as mature sized labeled proteins (Fig. 4, first and second lanes), indicating that they were imported and correctly processed. The identity of the second, 40-kDa band is not known. For deletion mutant Δ43–45 both precursor and a mature sized protein could be detected in the stromal fraction together with the 40-kDa band (Fig. 4, last lane). This indicates that this precursor is imported but processed less efficiently than full-length precursors. For deletion mutants Δ15–25 and Δ11–14 no mature sized protein could be detected in the stroma (Fig. 4, third and fourth lanes). However a 31-kDa stromal protein of unknown identity is labeled with Δ15–25 indicating that part of the precursor reaches the stroma. This is consistent with previous results (25), which show that these two mutants do not import or import with very low efficiencies.

Cross-linking during Binding—Next the effect of the deletions on the early steps of import was studied. Two conditions were used to generate intermediates in the initial stages of import: first conditions that allow only precursor binding, and second conditions that result in an early import intermediate. To generate binding conditions, chloroplasts were depleted of ATP by treatment with apyrase and nigericin and incubated with preferredoxin at 4 °C for 30 min. Under both conditions no envelope translocation takes place (33, 34).

The results of the cross-linking are shown in Fig. 5, A and B. Under binding conditions, cross-linking of the full-length wt-

**Fig. 2.** Cross-linking after import of the substrates for 20 min at 26 °C in the dark with 2 mM ATP. The molecular masses of standard proteins are indicated on the left of the figure. Image capture and analysis was performed on a phosphorimager. pref/d, preferredoxin.

**Fig. 3.** Identification of cross-linked envelope proteins. A, immunoprecipitation of cross-linked envelope proteins to the full-length precursor wt-S104 with antibodies specific to Toc86 (lane 2), Toc75 (lane 3), and Toc34 (lane 4). The starting material was loaded on lane 1. B, analysis of cross-linked envelope proteins to deletion mutant Δ15–25 with reversed phase chromatography. Total envelope proteins were applied to reversed phase chromatography corresponding to 10 mg of chlorophyll. A 45-ml acetonitrile gradient from 0–100% was used for the elution. Fraction numbers are indicated. S, starting material. White arrows indicate Tic20 and the 14-kDa protein. Western blotting verified the identity of Toc34, -75, and -86 (not shown). Similar results were obtained with cross-linked envelope proteins to the full-length precursor wt-S104 (not shown). The molecular masses of standard proteins are indicated on the left of the figure. Image capture and analysis was performed on a phosphorimager. pref/d, preferredoxin.
S104 containing a cross-linker in the transit sequence resulted in cross-linking of Toc86 as the major cross-linked product and Toc75 and Toc34 as minor cross-linked products (Fig. 5A, first lane). Some precursor proteins could also be detected because of intramolecular cross-linking. Cross-linking of the second full-length ferredoxin precursor, wt-S103, that did not contain a cross-linker in the transit sequence resulted in the labeling of Toc86 and faint labeling of Toc34 but no labeling of Toc75 (Fig. 5A, second lane). This could be because of the absence of a cross-linker in the transit sequence of wt-S103. When cross-linked as an early intermediate, for wt-S104 the major cross-linked product again was Toc86 and a minor one, Toc75 (Fig. 5A, third lane). The cross-linking pattern with wt-S103 was quite similar to wt-S104 (Fig. 5B, second panel, first lane). Cross-linking as an early intermediate with the full-length precursor resulted in a similar cross-linking pattern as after import; Toc34, -75, and -86 were the major cross-linking products. Also a 55-kDa band could be detected (Fig. 5B, second panel, first lane).

The cross-linking pattern after the binding of deletion mutant Δ43–45 resembled the cross-linking pattern of the wt-S103 control (Fig. 5A, last lane). Cross-linking of the early import intermediate (Fig. 5B, last lane) resulted in a lower ratio of Toc86/75, which could be because of the lower binding efficiency of this deletion mutant.

The cross-linking results with deletion mutants Δ11–14 and Δ15–25 contrasted strongly with the results obtained with wild-type, wt-S103, and wt-S104 precursors. Deletion mutants Δ11–14 and 15–25 cross-linked to Toc86 under binding conditions (Fig. 5A, third and fourth lanes) comparable with the full-length precursors. In contrast the cross-linking to Toc75 was much stronger and was about equal to the Toc86 intensity. Minor bands were found for Toc34 and the 55-kDa protein. When cross-linked as an early import intermediate, i.e. in the presence of ATP, essentially no cross-linked products were detected with deletion mutants Δ11–14 and Δ15–25 (Fig. 5B, third and fourth lanes); only Toc86 could be faintly labeled. These results indicate that the affinity of these two deletion mutants for Toc86 was decreased allowing an equilibration of cross-linking between Toc75 and Toc86. The reduced affinity for Toc86 apparently results in the release of the precursor from the import machinery in the presence of ATP.

The cross-linking results with deletion mutants

**DISCUSSION**

*In vitro* import experiments with deletion mutants in the ferredoxin transit sequence revealed a high information content of this targeting sequence (25). Most deletions interfere with the overall efficiency of import but different deletions identified separate domains within the transit sequence. The first, N-terminal domain mediates the initial binding. The second domain, the middle region of the transit sequence, is involved in envelope translocation after the initial recognition, and the third domain consisting of the C-terminal region is mainly involved in processing (25). The same domains were shown to be required for efficient import *in vivo* (26). In this study a label transfer cross-linking strategy was used to study
the interactions of the various deletion mutants in the transit sequence with components of the translocation machinery. The results of the cross-linking after the three different stages of import are summarized in Fig. 6 and compared with the previously obtained in vitro and in vivo import data (25, 26). The interactions observed between the full-length wild-type preferredoxin precursors and the import machinery were consistent with the results of similar cross-linking strategies using the precursor of the Rubisco small subunit (6, 9, 14).

The most C-terminal deletion used, Δ43–45, resulted in a cross-linking pattern similar to the full-length transit sequence. This mutant is processed inefficiently, as significant amounts of precursor-sized protein could be detected in the stroma after import. This illustrates that import and processing are not strictly coupled, as was shown before in vitro (26). The lower binding efficiency of deletion mutant Δ43–45 (25) is reflected by the lower affinity for Toc86.

A deletion in the central region of the transit sequence, represented by Δ15–25, resulted in under import conditions in a cross-linking pattern with the Toc components similar to the wild-type pattern, indicating normal interactions with the Toc components. Although Δ15–25 could not be imported, the precursor or part of it had crossed the outer membrane as judged from the labeling of Tic20, the 14-kDa protein, and a stromal protein. Therefore it can be concluded that the central region of the transit sequence is required for inner membrane translocation. These results also explain why deletion mutant Δ15–25 is a strong competitor for import although it cannot be imported. Different interactions, as compared with the wild-type, were observed under binding conditions in the absence or presence of ATP. The interaction with Toc86 is slightly decreased in the absence of ATP, whereas the labeling of Toc86 is much higher compared with the wild-type. One conclusion is that the affinity of Toc86 for Δ15–25 is lower than for the wild-type transit sequence and that under these conditions Δ15–25 is partially released from Toc86 to reach the next component of the import machinery such as Toc75. In the presence of ATP almost no cross-linked products could be detected. Apparently under these conditions no stable association with the import machinery could be established. After initial binding the deletion mutant Δ15–25 is pulled out of the import machinery instead of being transferred to the next stage of import. This result indicates that the central region of the transit sequence also contains motifs for recognition at the Toc components of the outer membrane.

Cross-linking of the N-terminal deletion mutant Δ6–14 resulted in hardly any cross-linked products, only under binding conditions and after import could minimal labeling of Toc86 be observed. This decreased labeling of Toc86 indicates that this component plays an important role in the initial stages of the import process, consistent with its proposed function (1, 2). Δ6–14 interacts with Toc86 but not with Toc75, which indicates that it cannot be released properly from Toc86. The N-terminal region of the transit sequence has a tendency to form an amphipathic helix in the presence of lipids (35). As this helix can not be formed in the deletion mutant Δ6–14, the formation of this helix may be important for the correct release of the precursor from Toc86. This is further supported by the finding that deletion mutant Δ6–14 cannot be inserted in monolayers mimicking the chloroplast outer membrane lipid composition (25). The cross-linking pattern of Δ11–14 was similar to the cross-linking pattern of deletion mutant Δ15–25. Thus for the same reason as with Δ15–25 the deletion mutant Δ11–14 is disturbed in the binding to Toc86 and the inner membrane interactions. In contrast to Δ15–25, Δ11–14 can be imported with a low efficiency. This suggests that the Δ11–14 deletion forms the overlap between the outer and inner membrane translocation domains having just enough information for full envelope translocation. The proposed functions of the different domains are indicated in the bottom panel of Fig. 6.

The whole transit sequence is important for the overall efficiency of binding and import (25, 26). This is the result of two separate aspects of the transit peptide: first the presentation of specific binding domains for the interaction with outer membrane components, as all deletion mutants had a different cross-linking pattern compared with the wild-type in the initial import reactions; and second the presence of different domains for interaction with the outer and inner membrane components of the transport machinery, as after import Δ6–14, Δ11–14, and Δ15–25 have distinct cross-linking patterns to inner and outer membrane components. In this study it is shown that region 6–14 of the transit sequence contains essential information for binding to the outer membrane component Toc86, as deletion mutant Δ6–14 almost completely fails to cross-link to Toc86. The region 15–25 also affects the interaction with Toc86 but to a lesser extent and mainly under nonimport conditions. In addition, this region contains information for interaction with components of the inner membrane, as indicated by the cross-linking of deletion mutant Δ15–25 to Tic20 and the 14-kDa protein. As deletion mutant Δ6–14 never reaches the inner membrane, no conclusion can be drawn for the importance of amino acids 6–14 for inner membrane interaction.

No region was found with specificity for interaction with Toc75. Deletion mutants Δ11–14 and Δ15–25 show increased binding to Toc75 under binding conditions, but we interpret this as the result of a decreased affinity for Toc86 rather than an increased affinity for Toc75. The information for association with the outer and inner membrane components therefore resides in two separate but partly overlapping domains in the first 25 amino acids of the transit sequence.

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