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Characterization of the Import Process of a Transit Peptide into Chloroplasts*

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In order to get insight into the functioning of transit sequences in chloroplast protein transport, the import of the full-length transit peptide of ferredoxin (trfd) was investigated. trfd rapidly associated with chloroplasts under import conditions and becomes protected against externally added proteases. Import of radiolabeled trfd is inhibited equally efficiently by nonlabeled trfd as well as by the intact precursor of ferredoxin. This strongly suggests that trfd enters the general import pathway of proteins into chloroplasts. trfd import was stimulated by ATP, which is the first demonstration that ATP is involved in membrane translocation of a targeting signal. Imported trfd was membrane-associated but was also partially degraded by internal proteases, most likely present in the stroma, indicating that the membrane-associated fraction of trfd is *en route* to its functional localization. The degradation products are exported out of the organelle. In contrast to the import of the precursor of ferredoxin, the import of trfd was independent of protease-sensitive components on the chloroplast surface, indicating that the initial binding of precursor proteins may be facilitated by transit sequence-lipid interactions.

The majority of the chloroplastic proteins is encoded on the nuclear DNA and synthesized in the cytosol. These proteins contain an N-terminal extension (1), the transit sequence that is necessary (2, 3) and sufficient (4) to import proteins post-translationally into chloroplasts (for review see Ref. 5). The import process is initiated by binding of precursor proteins to the chloroplast surface (6). Maximal binding requires the utilization of low amounts of ATP (100 μM) in the intermembrane space (7) and the presence of protease-sensitive components on the chloroplast surface (8). Binding can also be observed in the absence of ATP (9) and after protease treatment of chloroplasts (10), indicating that different binding stages and modes can exist. The subsequent translocation of the precursor proteins across the chloroplast envelope membranes requires a 1 mM ATP concentration (11, 12) in the stroma. Imported proteins are processed by a specific stromal protease (1, 13, 14) routed to their final localization within the chloroplast and assembled into holoenzymes.

Analysis of transit sequences reveals that there is little similarity in amino acid sequences (15). They are enriched in hydroxylated and small hydrophobic amino acids, have a positive charge, and lack acidic amino acids (16, 17). Despite the poor homology in the primary structure of transit sequences (15), they are able to perform their essential and specific functions in protein import processes (*i.e.* organelle-specific targeting, translocation across the envelope membranes, correct processing, and intraorganellar routing of precursor proteins).

This can be illustrated with the precursor of ferredoxin (prefd),[1] which follows the general import pathway (18). prefd is imported into the chloroplast stroma, where it is subsequently processed (11, 19). The apoprotein is converted into the biologically active holoprotein by insertion of the 2Fe–2S cofactor (20). Import of the largely unfolded prefd is independent of cytosolic factors (21), indicating that prefd itself contains all of the information for organelle-specific targeting and for the productive interaction with the import machinery leading to the translocation across the envelope membranes. Both processes require the presence of a functional transit sequence, because mature proteins do not bind to chloroplasts (22) and attachment of the ferredoxin transit sequence is sufficient to direct a foreign protein to the chloroplast stroma (23). The prefd transit sequence is also required for the interaction with the stromal processing enzyme, because deletions in the C-terminal region of the transit sequence strongly interfere with correct maturation of prefd (24). Very recently, it was demonstrated that prefd causes a transit sequence-dependent reduction in electrochemical resistance of the envelope in intact chloroplasts. The most likely interpretation of this phenomenon was that the transit sequence opens protein-conducting channels (25). How transit sequences function is completely unknown. However, it can be anticipated that they will exert specific interactions with components of the envelope membranes such as proteinaceous receptors (5) and envelope membrane lipids (26).

In order to get insight into the way transit sequences function, we studied the import of the transit peptide of ferredoxin (trfd) into chloroplasts. It is shown that trfd follows the ATP-dependent import pathway as is used by prefd. Import of trfd was independent of protease-sensitive components on the chloroplast surface. Imported trfd is rapidly degraded by internal chloroplast proteases followed by an efficient export of the degradation products.

MATERIALS AND METHODS

General—Dithiothreitol (DTT) and glutathione were obtained from Boehringer Mannheim. Sorbitol, Hepes, and bovine serum albumin

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[1] The abbreviations used are: prefd, precursor protein of ferredoxin; apofd, apoprotein of ferredoxin; HPLC, high performance liquid chromatography; trfd, transit peptide of ferredoxin; DTT, dithiothreitol; TCA, trichloroacetic acid; PAGE, polyacryamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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were from Sigma. Percoll and chromatographic equipment and materials were from Pharmacia (Uppsala, Sweden). Iodoacetamide was from Fluka (Buchs SG, Switzerland). All other chemicals were of the highest quality available.

Proteins—apofd was prepared out of holoprotein of ferredoxin as described (27). apofd was stored in aliquots in 150 mM Tris/HCl, pH 7.5, at a concentration of 1 mg/ml under nitrogen at −20 °C. prefd from *Silene pratensis* was purified as described by Pilon *et al.* (28). The protein was stored in aliquots at a concentration ranging between 1 and 1.5 mg/ml in 25 mM Tris/HCl, pH 7.6, 8 M urea, and 0.02% (v/v) β-mercaptoethanol under nitrogen at −20 °C.

[^{3}H]prefd was obtained by growing *Escherichia coli* BL21 cells (DE3) containing the vector pET11d in 35 ml of SV medium (containing per liter 28 g of ammonium ferrosulfate, 0.2 g of magnesium sulfate, 8 g of potassium dihydrogen phosphate, and 30 g sodium hydrogen phosphate with a pH of 6.9) at 37 °C with the following additions: 0.4% (w/v) glucose, 5 mg/liter thiamin, and 50 mg/liter ampicillin until the optical density was 0.6. Pelleted cells were resuspended in 5 ml of SV medium with 0.4% (w/v) glucose, 5 mg/liter thiamin, 50 mg/liter ampicillin, and 1 mM isopropyl-1-β-D-galactopyranoside. After 30 min, 1.0 mCi of [^{3}H]leucine (158 mCi/mmol) (Amersham Corp.) was added, and the cells were allowed to grow for another 3 h. [^{3}H]prefd was purified as described (28), except that the cells were lysed by sonication (Branson) and a smaller gel filtration column (1.5 × 40 cm) was used. Protein concentrations were determined according to Bradford (29) with bovine serum albumin as reference.

Transit Peptide of Ferredoxin—A 47-mer corresponding to the transit sequence of ferredoxin from *S. pratensis* was synthesized on an Exell Pepsynthesizer by Millipore (Watford, UK). trfd with the sequence ASTLSTLSVSASLLPKQQPMVASSLPTNMGQALFGLKAGSRGRVTAM differs only from the sequence deduced from the gene (30) by the absence of the N-terminal methionine, which is post-translationally removed in the cytosol and was blocked at the C terminus with an amide group to avoid the negative charge at this position. trfd was purified by reversed phase high performance liquid chromatography (HPLC) as described (26). The purity of the peptide was estimated to be over 98%, as determined by analytical HPLC. The identity of trfd was confirmed by N-terminal sequencing of 20 amino acids by Edman degradation as described (28) and by quantitative amino acid analysis. Peptide concentrations were determined by the bicinchoninic acid protein assay (Pierce) using bovine serum albumin as reference.

Purified trfd was labeled by reductive methylation using [^{14}C]formaldehyde (31). In short, 2 mg of trfd (0.47 μmol) dissolved in 500 μl of distilled water was added to 500 μl of 20 mM Hepes, pH 7.6, containing 62.5 μmol of sodium cyanohydrogen boride ($NaCNH_3B$) and 3.75 μmol of [^{14}C]formaldehyde (59 mCi/mmol) (Amersham Corp.) and was incubated for 1.5 h under nitrogen with constant mixing. After the addition of another 62.5 μmol $NaCNH_3B$ and further incubation of 1.5 h, trfd was precipitated by 10% (w/v, final concentration) trichloric acid (TCA). The trfd precipitate was washed 3 times with ice-cold acetone and, after evaporation of the acetone, dissolved in 600 μl of distilled water to a concentration of 2 mg/ml, divided into aliquots, and stored under nitrogen at −20 °C. The ^{14}C-labeled transit peptide of ferredoxin could be visualized as a single band by Tricine/SDS-PAGE (32) followed by fluorography. It was shown that all applied radioactivity was present in the peptide band. The specific activity of [^{14}C]trfd was 49 mCi/mmol, and trfd contained 0.8 [^{14}C]methyl group per molecule.

Import Experiments—Chloroplasts were isolated out of 10–12-day-old pea seedlings cv. Feltham First as described (33). Import reactions (28) took place in a buffer consisting of 330 mM sorbitol, 50 mM Hepes/KOH, pH 8.0, 200 μg/ml antipain, 1 mM DTT, and 2 mM Mg-ATP (import buffer), unless indicated otherwise. Import mixtures with a volume of 300 μl were used containing chloroplasts to an equivalent of 60 μg of chlorophyll and trfd and prefd, as indicated. Chloroplasts were added to (poly)peptide containing import mixtures. Import experiments were carried out at under import conditions (*i.e.* 25 °C in the light for 20 min) unless indicated otherwise. After the import experiment, 1 ml of ice-cold import buffer was added to stop the import process, and the samples were divided into two fractions. In one fraction, the chloroplasts were reisolated, washed, and analyzed by liquid scintillation counting and gel electrophoresis. This fraction contained both imported and bound trfd and prefd molecules. The other fraction was incubated for 15 min at 4 °C with 7.5 μg of thermolysin to digest (poly)peptides bound to the chloroplast surface (34) and subsequently treated as above, yielding the amount of trfd and prefd imported into the chloroplasts. The chloroplast recovery was determined by measuring the total amount of protein according to Bradford (29). The influence of DTT on prefd and trfd import was investigated by import experiments in the presence of DTT concentrations ranging from 0 to 1 mM.

Because chlorophyll interferes with the analysis of trfd by Tricine/SDS-PAGE (32), the peptide was precipitated by 80% acetone, followed by centrifugation for 5 min at 14,000 rpm. The supernatant, which did not contain trfd as was verified by liquid scintillation counting, was removed, and traces of acetone were evaporated. The pellet was resuspended in 6 M urea, 10 mM Tris/HCl, pH 7.6, and 2 mM DTT by sonication for 15 min in a bath sonicator. Samples containing prefd were analyzed directly by SDS-PAGE according to Laemmli (35). Protease pretreated chloroplasts were obtained by incubation of chloroplasts equivalent to 1 mg of chlorophyll with 250 μg of thermolysin for 20 min at 4 °C in the dark. Subsequently, the chloroplasts were reisolated by centrifugation through a preformed 50% Percoll gradient containing 2 mM EDTA in order to block the thermolysin activity. Chloroplast fractionation was performed by hypertonic lysis in 10 mM Hepes, pH 8.0, followed by a centrifugation for 30 min at 60,000 rpm in a Beckman TLA 100.3 rotor. The membrane pellet was resuspended for further analysis. In order to decrease the sample size of the supernatant fraction, the proteins were precipitated by 80% acetone. The pellet was resuspended in 6 M urea, 10 mM Tris/HCl, pH 7.6, and 2 mM DTT by sonication for 15 min in a bath sonicator.

Determination of trfd Recovery and Stability—After incubation under import conditions, unless indicated otherwise, of 2.5 μg of [^{14}C]trfd in 300 μl of import buffer containing chloroplasts to an equivalent of 60 μg of chlorophyll, proteins were precipitated by incubation with TCA (final concentration, 10%, w/v) for 15 min at 0 °C. Intact trfd is quantitatively precipitated under these conditions. The protein pellet was resuspended in 300 μl in 100 mM Tris/HCl, pH 7.6, 8 M urea, and 20 mM DTT by sonication. The percentage of radioactive label present in the pellet and supernatant was determined by liquid scintillation counting. In order to localize trfd and trfd degradation products after the incubation, the chloroplasts were pelleted and resuspended. In both, the resuspended chloroplast pellet and supernatant proteins were precipitated by TCA, and the fraction of precipitable radioactivity was determined. In some experiments, chloroplasts were lysed in import buffer with a sorbitol concentration of 80 mM.

trfd Vesicle Binding—In order to investigate whether binding of trfd to lipid domains could result in protease protection of trfd, binding experiments of trfd (2 μg added) to large unilamellar vesicles of 200 nmol of lipid composed of a lipid extract of the chloroplast outer envelope membrane were performed according to Ref. 36. Vesicles containing associated trfd (0.29 μg) separated from nonassociated trfd by centrifugation were incubated for 15 min with 7.5 μg of thermolysin at room temperature.

RESULTS

The ability of [^{14}C]trfd to associate with chloroplasts was investigated under conditions where prefd is imported and correctly processed in the light at 25 °C in a buffer containing 2 mM ATP (28). Analysis by Tricine/SDS-PAGE of reisolated and washed chloroplasts from the incubation mixtures showed that in time increasing amounts of trfd became stable associated with the organelle (Fig. 1*A*).

To investigate whether chloroplast associated trfd was bound to the chloroplast surface, the incubation mixtures were treated with thermolysin, which is not able to enter the chloroplast intermembrane space and which can only digest proteins that are present on the chloroplast surface (34). Interestingly, the majority of the associated trfd was not degraded (Fig. 1*B*). In control experiments, comparable amounts of trfd in the import buffer were digested within 30 s by identical amounts of thermolysin (data not shown). Furthermore, trfd associated to large unilamellar vesicles with a lipid composition comparable with the chloroplast outer envelope membrane was found to be completely digestable by thermolysin (data not shown). This indicated that binding to lipid surfaces does not result in protection against proteases. It can therefore be concluded that trfd had reached a protease-protected position, which we define as "import." Quantification of this time course experiment (Fig. 1*C*) shows that association and import of trfd is a linear process in time.

The addition of increasing amounts of labeled trfd to isolated intact chloroplasts under import conditions led to an increased

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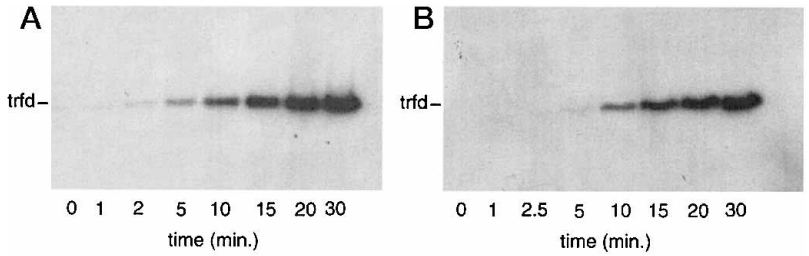

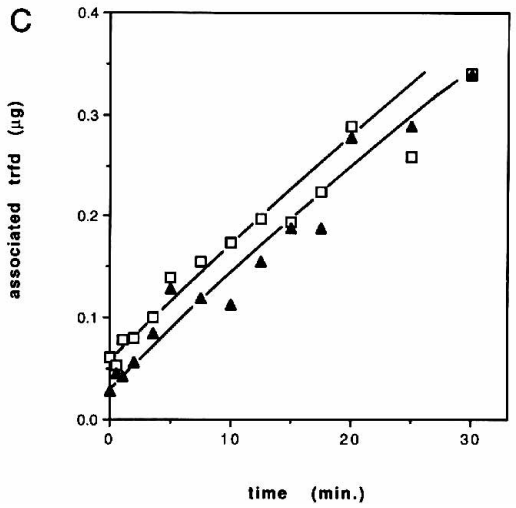

FIG. 1. **Time course of import of [^{14}C]trfd.** *A*, total chloroplast-associated trfd. *B*, protease-protected chloroplast-associated trfd. *C*, quantitative representation of *A* and *B*. □, total associated trfd; ▲, protease-protected trfd. 4 μg of trfd is used per incubation. In *A* and *B*, trfd is visualized by Tricine/SDS-PAGE and fluorography.

association of trfd to chloroplasts (data not shown). Association and import of [^{14}C]trfd is saturable. Moreover, association and import are tightly coupled over a large range of transit peptide concentrations. From these results it can be calculated that maximal 16 ± 2 × 10^3 trfd molecules/minute/chloroplast are imported assuming that 30 μg of chlorophyll corresponds to 4.5 × 10^7 chloroplasts (21). The value of the V_{max} of trfd import is close to the value of the V_{max} of 22 × 10^3 molecules/minute/chloroplast reported for prefd import (37).

Quantification of the experiment shown in Fig. 1 showed that nearly all chloroplast-associated trfd was present in the trfd band (data not shown). Fractionation of reisolated chloroplasts from incubation mixtures revealed that all chloroplast-associated radioactivity is localized in the membrane fraction (Fig. 2*A*). These observations do not exclude the possibility that part of the transit peptide is degraded during or after import. That this may be the case is suggested by the observation that the transit sequence cleaved off from imported prefd could not be detected in the membrane fraction nor in the soluble fraction, although it contains 7 of the 13 [^{3}H]leucine residues (Fig. 2*B*). This demonstrates that the transit sequence is rapidly digested after processing. It should be realized that processing of prefd cannot be observed by Tricine/SDS-PAGE as used in Fig. 2*B*, because this gel system does not separate prefd from holoprotein of ferredoxin. However, control experiments using SDS-PAGE demonstrated that prefd was correctly processed under the experimental conditions (data not shown).

To get direct insight into possible trfd degradation within chloroplasts, TCA precipitation experiments were done (Fig. 3). Intact trfd in import buffer without chloroplasts (Fig. 3, *lane 1*) or with chloroplasts in conditions under which no import can take place (Fig. 3, *lane 2*) can be nearly quantitatively precipitated by TCA. In contrast, a large fraction of trfd incubated with lysed chloroplasts is not precipitable due to digestion by proteases released from the chloroplasts (Fig. 3, *lane 3*). Under import conditions a substantial fraction (13 ± 1%) of the added ^{14}C radioactivity is nonprecipitable (Fig. 3, *lane 4*), which demonstrates that indeed part of the added trfd is degraded, like in case of transit sequence liberated from the import precursor. This is most likely due to digestion inside the chloroplasts, but in principle this could also, in part be due to digestion by proteases liberated from chloroplasts during the incubation. To get an estimate of the maximal contribution of such released proteases, trfd was incubated in the supernatant of chloroplasts preincubated under import conditions. This leads to substantially less (7 ± 1%) degradation (Fig. 3, *lane 5*). Thus, it has to be concluded that at least 6% of the added trfd is degraded by internal chloroplast proteases. This has to be compared with 15% of the added trfd, which is associated as intact trfd to chloroplasts under import conditions (Table I).

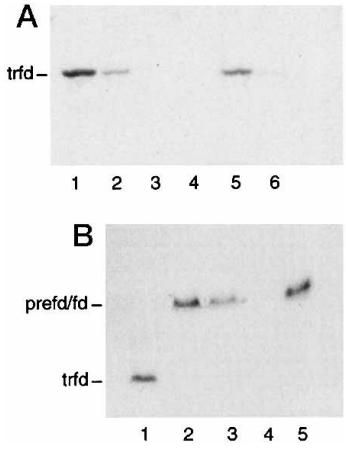

FIG. 2. **Intrachloroplast localization of imported trfd and prefd.** *A*, localization of trfd. *Lane 1*, total associated trfd; *lane 2*, imported trfd; *lane 3*, soluble fraction; *lane 4*, protease-treated soluble fraction; *lane 5*, membrane fraction; *lane 6*, protease-treated membrane fraction. *B*, localization of prefd. *Lane 1*, trfd standard; *lane 2*, total associated prefd; *lane 3*, imported prefd; *lane 4*, membrane fraction; *lane 5*, soluble fraction. Intensities of (poly)peptide bands cannot be directly compared because different lanes do not necessary contain the same amount of chloroplasts.

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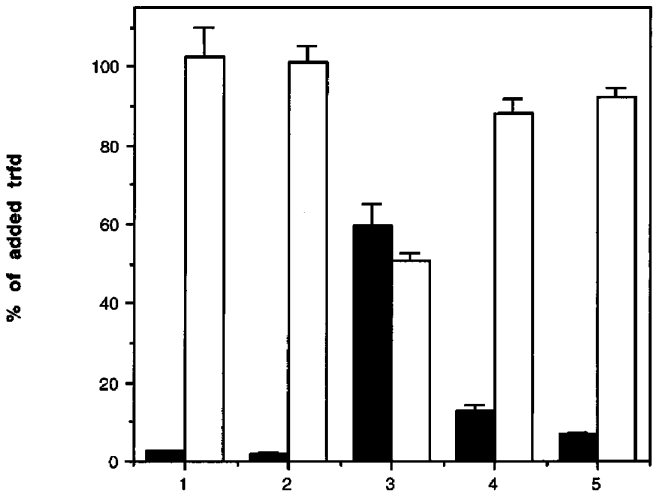

FIG. 3. **Determination of the recovery of intact trfd after import into chloroplasts.** After incubation for 20 min under import conditions, proteins are precipitated by TCA, and the percentage of precipitable and nonprecipitable counts, expressed as the percentage of the total amount of added trfd, is determined by liquid scintillation counting. For each experiment, 2.5 μg of trfd was used. Mean values and standard deviations of three independent measurements are shown. *Lane 1*, control experiment, trfd incubated in import buffer in the absence of chloroplasts; *lane 2*, incubation of trfd with chloroplasts in conditions under which no import take place (in the dark without ATP at 20 °C); *lane 3*, incubation of trfd in import buffer with lysed chloroplasts; *lane 4*, import experiment, trfd incubated in import buffer in the presence of chloroplasts under import conditions; *lane 5*, incubation of trfd in the supernatant of preincubated chloroplasts under import conditions. *Open bars* represent TCA-precipitable counts; *closed bars* TCA-nonprecipitable counts.

TABLE I
Localization of trfd degradation products generated by internal chloroplast proteases

After incubation of trfd under import conditions, the incubation mixtures were centrifuged. In both the resuspended chloroplast pellet and supernatant, the proteins were precipitated by TCA, and the percentages of precipitable and nonprecipitable counts, expressed as the percentage of the total amount of added trfd, were determined by liquid scintillation counting. Mean values and standard deviations of three independent measurements are shown.

	Chloroplast pellet	Supernatant
TCA-precipitable	15.1 ± 3.9	68.9 ± 5.4
TCA-nonprecipitable	2.0 ± 0.2	13.9 ± 2.8
Total	17.1 ± 3.9	82.8 ± 6.2

In order to determine the localization of the degradation products of trfd, intact chloroplasts were isolated by centrifugation after the incubation. In both the resuspended pellet and the supernatant, the percentage of intact and degraded trfd was determined by TCA precipitation. Table I demonstrates that under import conditions virtually all chloroplast-associated trfd is intact (TCA-precipitable) and that the degradation products (TCA-nonprecipitable) are present in the chloroplast supernatant. It thus has to be concluded that the trfd degradation products generated by internal chloroplast proteases are rapidly exported.

trfd competes for import of prefd (27). Fig. 4 shows that the reverse is also true. Unlabeled trfd and prefd equally efficiently inhibit the import of [^{14}C]trfd, indicating that prefd and trfd compete for the same limiting import step. Competition is a specific process depending on the transit sequence because apofd is not able to inhibit trfd import (Fig. 4).

Binding and import of precursor proteins into chloroplasts require ATP as energy source (5). ATP also affects chloroplast association and import of [^{14}C]trfd (Fig. 5). In the absence of exogenous ATP, trfd already displays some association and

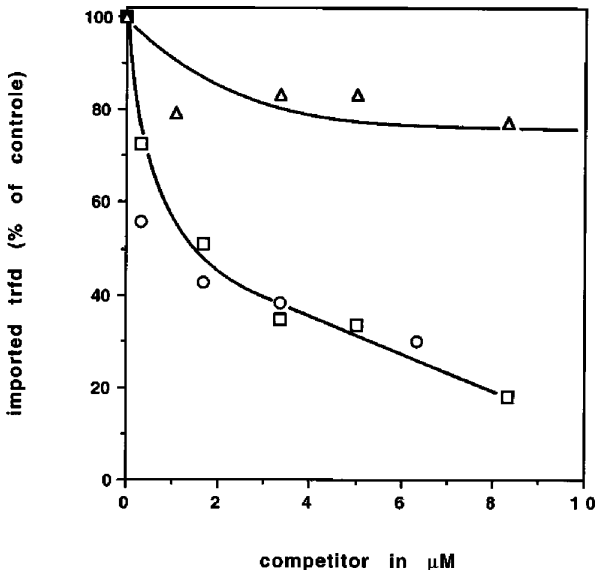

FIG. 4. **Inhibition of [^{14}C]trfd import by trfd, prefd, and apofd.** Competition by trfd (□), prefd (○), and apofd (△). 1 μg of [^{14}C]trfd is used per incubation mixture of 150 μl containing 30 μg of chlorophyll.

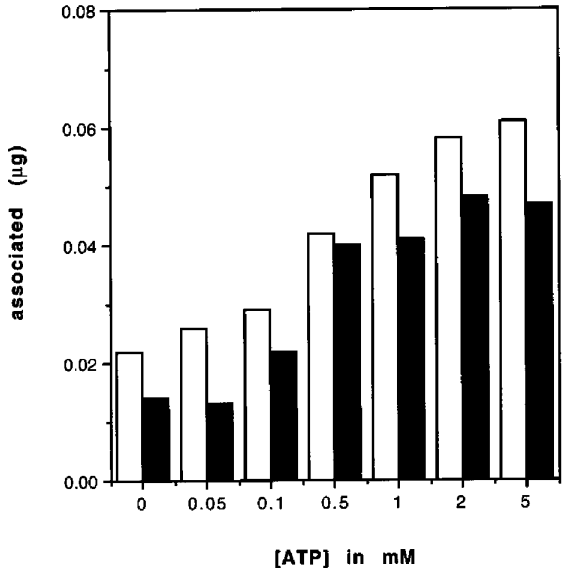

FIG. 5. **ATP dependence of trfd import.** *Open bars*, total associated trfd; *closed bars*, imported trfd. 4 μg of trfd is used per incubation. Incubation mixtures contained 200 nM nigericine in order to prevent intrachloroplast ATP production. The import experiment is performed at 4 °C under green light. Chloroplasts are incubated for 30 min at 0 °C in the presence of 200 nM nigericine in the dark to deplete them of ATP.

import into chloroplasts. However, increasing the ATP concentration strongly stimulates both trfd association and import, indicating that ATP-consuming proteinaceous components are involved in trfd import. trfd association and import is maximal around 1–2 mM ATP, which is very similar to the ATP concentration of 1 mM at which prefd import is maximal (21).

Digestion of proteinaceous components localized on the chloroplast surface by thermolysin reduces the import of precursor proteins into chloroplasts (33), as is shown for prefd in Fig. 6. In contrast, the import of trfd is hardly affected by protease pretreatment.

DISCUSSION

The aim of this study was to investigate the functioning of transit sequences in chloroplast protein import. The approach was to study the import of the full-length transit

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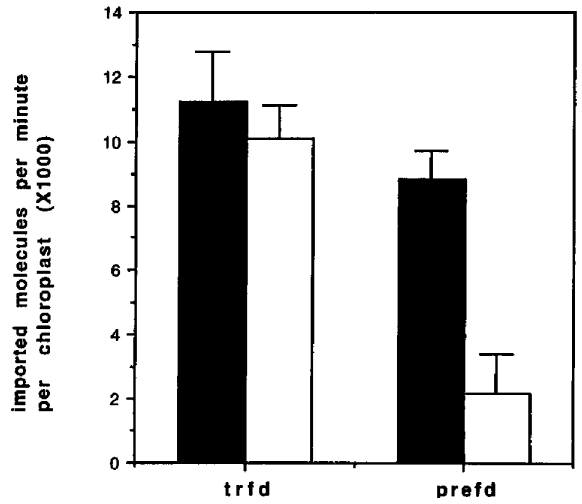

FIG. 6. **Effect of chloroplast protease pretreatment on trfd and prefd import.** *Closed bars*, control chloroplast; *open bars*, protease-pretreated chloroplasts. Mean values and the standard deviation of three individual experiments are shown. 1 μg of [^{14}C]trfd (0.2 μmol) and 2.34 μg of [^{3}H]leucine-prefd (0.16 μmol) are used per incubation mixture of 150 μl containing 30 μg of chlorophyll.

peptide of ferredoxin.

It was shown that trfd enters the general import pathway of proteins into chloroplasts. This conclusion is based on the following observations. First, associated trfd is largely protected against externally added protease, indicating that trfd has reached an internal chloroplast localization. Second, [^{14}C]trfd import is equally efficiently inhibited by nonlabeled trfd and prefd. Third, trfd inhibits the import of prefd into chloroplasts (27). Fourth, trfd import is saturable and occurs with a maximal velocity close to the value of the V_{max} of prefd import (37). Finally, trfd import is stimulated by ATP.

This is the first example of a targeting signal for which translocation across a membrane is stimulated by ATP. This differs strikingly from the situation in mitochondria where the translocation step of the presequence is driven by the membrane potential ($\Delta\psi$) across the inner membrane (38–41). This difference in the energy required for protein translocation into chloroplasts and mitochondria demonstrates that the mechanisms of protein import into both organelles are fundamentally different. The stimulation of trfd import by ATP can, for instance, be due to interactions of trfd with membrane-associated chaperonines and to the fact that ATP is consumed in transit sequence-chaperone binding/release steps. From a comparison of transit sequences, it was postulated that they are unstructured, which makes them prone to interact with chaperonines (42).

After import, protease-protected intact trfd could be identified solely in the membrane fraction. The absence of trfd in the soluble fraction, which mainly consists of the stroma, could indicate that trfd import is halted at the level of the envelope membranes. This would suggest that interaction of the mature region of precursor proteins with the import machinery is required to complete the import into the stroma. The observation that fusion proteins, for instance consisting of the ferredoxin transit sequence and the yeast mitochondrial manganese superoxide dismutase (23), are correctly imported into the stroma suggests that this presumed interaction should be rather aspecific. Alternatively, and more likely, the absence of trfd in the stroma could be due to digestion of trfd during or after import. In agreement with this proposal, it was observed that part of the chloroplast-associated trfd was digested by internal chloroplast proteases. This protease activity was not associated to the chloroplast surface because incubation of trfd with chloroplasts, under the condition that no import could take place, resulted in only a marginal trfd degradation. Instead, the degradation was shown to be largely due to internal proteases. This protease activity could be present in the stroma, because the cleaved transit sequence after import of prefd into the stroma could not be detected in the membrane nor in the soluble fraction. The proposed degradation of trfd in the stroma indicates that the membrane-associated protease-protected trfd is *en route* to its functional localization.

The degradation of transit sequences after import may be required to prevent the accumulation of large amounts of transit sequence, which may very well have a poisoning effect. For instance, the surface-active and membrane-seeking properties of transit sequences (26) could lead to membrane insertion of large amounts of transit sequences, affecting membrane functioning. Furthermore, this result strongly argues against a second long-lived function of transit sequences in chloroplasts.

Surprisingly, although trfd was degraded by internal protease, the degradation products were almost entirely present in the external chloroplast medium. Therefore, the degradation products should be exported out of the chloroplast by a so far unknown mechanism. This transport process could enable the reuse of transit sequence degradation products in the cytosolic protein synthesis.

trfd is imported into chloroplasts along the general import pathway of the precursor protein; therefore differences in import characteristics of trfd and prefd may be related to differences in import requirements of transit sequences and mature part of precursor proteins. One striking difference between trfd and prefd import was the independence of trfd import to protease-sensitive components on the chloroplast surface. Also, the import of outer envelope membrane proteins was shown to be independent of protease-sensitive components on the chloroplast surface (44–46), but these proteins likely follow an alternative pathway (44). Therefore, the protease-sensitive components of the chloroplast surface seem not to be involved in trfd binding and import. This suggests that trfd initially binds to the chloroplast surface by interactions with the membrane lipids. This hypothesis is supported by the observation that prefd inserts, via its transit sequence, efficiently and specifically in lipid monolayers composed of a lipid extract of its target membrane (26) and binds to lipid vesicles (36). Transit sequence-lipid interactions may result in the insertion of the transit sequence in lipid domains (36), enabling the diffusion of precursors to the import machinery in a two-dimensional way, which will be more efficient than via three-dimensional diffusion through the aqueous phase. Besides this, transit sequence-lipid interactions result in the induction of secondary structures in the otherwise unstructured transit peptide, which may function as recognition motive for the import machinery (47) Furthermore, these interactions can result in reorientation of lipid molecules (48). This change in lipid organization can directly be involved in protein import (49) or be required for the activation of the import machinery.

Comparison of the dissociation constants reveals that trfd binds with a 30-fold lower affinity to lipid vesicles than a precursor protein to chloroplasts (22, 36). This suggests that the initial binding to the lipids is followed by an interaction with proteinaceous components of the import machinery.

Recent studies (50–53) have identified several of these proteinaceous components. Schnell *et al.* (52) and Kessler *et al.* (53) identified six envelope membrane proteins associated to a translocation intermediate. Two of these proteins, of 34 and 86 kDa, are both integral outer envelope membrane proteins and are supposed to be exposed to the cytosol, due to their sensi-

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tivity to externally added proteases. Because trfd import is independent of protease-sensitive components on the chloroplast surface, it is unlikely that the 34- and 86-kDa proteins directly interact with the transit sequence and are involved in precursor protein targeting. Subsequently, it is unlikely that these proteins function as proposed by Kessler *et al.* (53) in the regulation of the presentation of transit sequences to the import machinery or by regulating the opening of the translocation channel. The 34- and 86-kDa proteins are most likely required for the import of the mature region of the precursor, and can perform the following functions. First, by interacting with the mature part region of the precursor, they could stabilize the binding of precursors to the chloroplast surface. Second, they may be required for a productive interaction of the precursor with other components of the import machinery. Finally, they may be required to bring the precursor in an import-competent conformation, for instance by reduction or by unfolding of mature regions of precursor proteins. In case of prefd, they could act as reductases because prefd import is stimulated by DTT (21), whereas trfd import was independent of the DTT concentration (data not shown). Guérra *et al.* (43) observed the unfolding of a precursor protein when incubated with outer envelope membrane vesicles, but whether this activity was protein- or lipid-mediated is not known.

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