Precursors with Altered Affinity for Hsp70 in Their Transit Peptides Are Efficiently Imported into Chloroplasts*

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Protein import into chloroplasts is postulated to occur with the involvement of molecular chaperones. We have determined that the transit peptide of ferredoxin-NADP(H) reductase precursor binds preferentially to an Hsp70 from chloroplast stroma. To investigate the role of Hsp70 molecular chaperones in chloroplast protein import, we analyzed the import into pea chloroplasts of preproteins with decreased Hsp70 binding affinity in their transit peptides. Our results indicate that the precursor with the lowest affinity for Hsp70 molecular chaperones in its transit peptide was imported to chloroplasts with similar apparent $K_m$ as the wild type precursor and a 2-fold increase in $V_{max}$. Thus, a strong interaction between chloroplast stromal Hsp70 and the transit peptide seems not to be essential for protein import. These results indicate that in chloroplasts the main unfolding force during protein import may be applied by molecular chaperones other than Hsp70s. Although stromal Hsp70s undoubtedly participate in chloroplast biogenesis, the role of these molecular chaperones in chloroplast protein translocation differs from the one proposed in the mechanisms postulated up to date.

Plastids accomplish a great variety of metabolic functions and developmental roles in plants and eukaryotic algae. They are distributed in different amounts in each plant tissue but are present in almost all plant cells. These organelles contain their own genomes, but most of their proteins are nuclear encoded and post-translationally imported through a complex machinery located in the plastid envelope. The import process requires a number of cytosolic and stromal soluble factors and several membrane-bound and integral proteins. Most of these proteins have been identified and studied (1–3). The significant biochemical and molecular data achieved to date have allowed the creation of working models for the chloroplast protein import mechanism.

Precursor proteins containing N-terminal signal sequences are bound to the chloroplast outer membrane probably by interacting initially with lipids and then, by a “docking” step that involves a highly specific protein-protein recognition, and GTP-ATP hydrolysis and/or exchange (4). The passage of the preprotein through the membranes is initiated by its N terminus. Once the precursor protein has reached the irreversible stage recognized as “early import intermediate,” the transit peptide and the N-terminal unfolded portion of the preprotein pass through the outer membrane channel and interact with some of the components of the internal membrane translocation apparatus (5–7). The complete translocation of the protein requires high energy availability (>1 mtr ATP in vitro import experiments) to unfold the bound folded precursor and move it across both envelope membranes (8, 9). Finally, the transit peptide is proteolytically removed (10), and the mature protein is folded to its final conformation.

Two possible mechanisms have been proposed for mitochondrial protein import (11–14). In both models, internal Hsp70s has been postulated to be the main motor of the process. The Brownian ratchet mechanism assumes that the precursor chain diffuses within the translocation pore. Then, Hsp70 molecular chaperones located in the inner side of the organelle bind to sites distributed along the polypeptide chain hampering the reverse movement of the polypeptide, thus promoting an unidirectional movement of the chain into the mitochondrial matrix (13, 14). The second model postulates that Hsp70 associates with the membrane-bound complex (Sec63p or Tim44) and the precursor protein. Hsp70 then undergoes an ATP-dependent conformational change that pulls the precursor through the pore, driving the external unfolding, and impelling the precursor protein to cross the membranes (11, 12, 15). The interaction of the transit peptides of mitochondrial and chloroplast precursors with Hsp70, which helps to sustain the above mentioned models, is supported by experimental and theoretical means (14–21).

The Hsp70 molecular chaperones comprise a protein family that is widely distributed among different organisms and subcellular compartments. Several Hsp70 proteins were found to be located in chloroplasts, associated with the envelope, or resident in the stroma. Com70 was detected facing the cytoplasm, Hsp70-IAP was found to be exposed to the intermembrane space between the outer and inner membranes, and at least two more Hsp70 molecular chaperones are located in the stroma, CSS1, for which its cDNA has been isolated and sequenced, and another Hsp70 less characterized (22–24). The intermembrane and stromal Hsp70s can be considered ideal candidates for providing the driving force during precursor protein import into chloroplasts. However, it is still a matter of discussion which class of chaperones acts as the main molecular motor of the protein import into chloroplasts. It has been proposed that an Hsp93 molecular chaperone (ClpC), a member...
of the Hsp100 family associated with the inner face of the envelope alone or in collaboration with a plastidic Hsp70, may function as the driving force of the system (4, 25, 26).

In previous work, we have demonstrated that more than 75% of chloroplast transit peptides have at least one putative DnaK (Escherichia coli Hsp70 molecular chaperone) binding site. We have also confirmed that an interaction exists between the transit peptide of ferredoxin-NADP(H) reductase precursor (preFNR) and Hsp70 molecular chaperones from plant cells in vitro (21). Similar observations were made for the transit peptide of the small subunit of Rubisco (18).

In this report we have studied the import of wild type and mutant precursors with altered affinity for Hsp70 in their transit peptides into isolated chloroplasts and their interactions with cytosolic and chloroplast Hsp70 molecular chaperones. As a model, the ferredoxin-NADP(H) reductase (FNR) precursor was used. This enzyme catalyzes NADP+ reduction during photosynthesis. The NADPH generated is used for CO2 fixation and other biosynthetic pathways (27). preFNR had been previously purified as a tightly folded precursor containing non-covalently bound FAD. The protein was fully active and was efficiently imported to intact pea chloroplasts (28). We used monoclonal and polyclonal antibodies to show that the transit peptide of preFNR interacts preferentially with the Hsp70 molecular chaperone CSS1 and not with chaperones from the cytosol. We performed quantitative protein import studies using precursors with decreased Hsp70 binding ability in their transit peptides to challenge the current working models on polypeptide translocation. Here we show that weakening the interaction between the chaperone and the transit peptide does not impair the protein import process. To the contrary, mutations introduced in the transit peptides produced a 2-fold import efficiency increase, whereas they did not affect the precursor binding to the import machinery. Therefore, in chloroplasts the main unfolding force may be applied by molecular chaperones different from stromal Hsp70s, and a simple Hsp70-Brownian ratchet mechanism may not be sufficient to drive chloroplast polypeptide import. These results provide additional insights to the process and are discussed under the light of the current models.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions, Expression, and Purification of preFNRs—**

The cDNAs coding for preFNR variants (preFNR-14, preFNR-23, and preFNR-1234) were inserted into a modified pET32 vector (Novagen) and expressed in E. coli as thioredoxin N-terminal fusion proteins. The construction of this modified vector was previously described (29). Then, the pStl-EcoRI cDNA fragments (=1.3 kbp) coding for pea preFNR mutants from plasmids pGFF202-14, pGFF202-23, and pGFF202-1234 (21) were subcloned into the modified pET32b vector digested with PstI and EcoRI. To ensure that no sequence artifacts were introduced during protein expression E. coli BL21(DE3)/pLysS cells were transformed each time with the different sequenced plasmids and then grown in Luria-Bertani medium containing antibiotics at 37 °C up to the suspension reached an absorbance of 0.7 at 600 nm. Expression and purification of [35S]labeled preFNRs were performed as described previously (29).

**Binding of Hsp70 Chaperones to GST-TP Fusion Proteins—**

In vitro binding of plant Hsp70 chaperones to GST-TP or GST-TP-1234 was performed essentially as described (21), using cytosol or chloroplast extracts. Briefly, GST-TP fusion proteins used for these studies were expressed and purified from E. coli strain BL21 (DE3)/pLysS. The proteins were incubated with 8 M urea for 10 min after the translation midpoints were estimated from the continuity of the gel bands, which depends upon the rate of unfolding. The observed distributions of protein at the midpoint were compared with those predicted for different arbitrary values of the unfolding rate constants (34).

**Analytical Procedures—**

Quantification of labeled protein bands was performed using Gel-Pro Analyzer Software 3.1 (Media Cybernetics). Different amounts of purified labeled protein were included in each experiment as standard. Import rates and kinetics parameters were determined by fitting the data obtained to a first order equation using Sigmaplot. FNR-dependent diaphorase activity was determined by published methods (35) using ferricyanide reduction at 420 nm (ε420 = 1 mmol cm−1).

**Table 1**

| Protein          | Binding Affinity (±SD) | Import Efficiency (±SD) |
|------------------|------------------------|-------------------------|
| wild type GST-TP | 0.7 ± 0.2 nmol/200 µl   | 0.8 ± 0.1%               |
| preFNR-1234      | 0.3 ± 0.1 nmol/200 µl   | 0.6 ± 0.1%               |
| preFNR-23        | 0.5 ± 0.2 nmol/200 µl   | 0.7 ± 0.1%               |

**Figure Legends**

1 The abbreviations used are: preFNR, ferredoxin-NADP(H) reductase precursor; FNR, ferredoxin-NAD(P)H reductase (EC 1.18.1.2); GST, glutathione S-transferase (EC 2.5.1.18); GST-TP, GST-tp transit peptide fusion protein; Ribosucrose, ribulose-bisphosphate carboxylase/oxygenase.
**RESULTS**

**Purification of FNR Precursors Containing Mutations in Their Transit Peptides**—Four amino acid substitutions were successfully introduced previously into the preFNR transit peptide sequence by PCR techniques (Fig. 1A, see also Ref. 21). Mutations were designed to produce a major increase of $\Delta \Delta G_k$ for each region, thus decreasing the putative E. coli Hsp70 (DnaK) binding with a minimal sequence variation. It was experimentally determined that transit peptides carrying these mutations exhibit an increased release of bound DnaK. Moreover, the effect introduced by each mutation was accumulative (21). It has been stated that predicted binding specificity for E. coli Hsp70 could be valid for a broad range of Hsp70s (20, 21). Moreover, the above mentioned mutations were proved to affect the interaction of the preFNR transit peptide with plant Hsp70 chaperones (21).

Three different preFNRs carrying the mutations described above and the wild type precursor were cloned as thioredoxin N-terminal fusion proteins, expressed in E. coli, and purified by affinity chromatography using a nickel-nitritotriacetic acid-agarose resin. Precursor proteins were released of Hsp70 molecular chaperones as described previously (36). Then, precursors were separated from thioredoxin by digestion with the endoprotease thrombin. Purified samples are shown in Fig. 1B. Precursor preparations used were >90% pure as judged by SDS-PAGE electrophoresis and scanning densitometry. Faint minor bands were reactive against anti FNR antibodies. They could be truncated proteins or the result of some proteolytic digestion of the fusion protein. No Hsp70 molecular chaperones were detected by the monoclonal and polyclonal anti-Hsp70 antibodies used in this work copurifying with the isolated precursor proteins.

**Interaction of Stromal Hsp70s with Precursor Proteins**—Models of post-translational protein translocation assume that interaction of the incoming polypeptide with the chaperones responsible for its import occurs only at the luminal side of the endoplasmic reticulum, or the inner side of the organelles (37–39). Considering that homologs of the Hsp70 chaperone family are present at both sides of the chloroplast envelope, differences in affinities or specificities should be required at both sides of the membranes to uphold these models of protein import. Thus, we have analyzed the binding preference of the preFNR transit peptide for molecular chaperones from cell cytosol or chloroplast stroma. In the case of cellular extracts, chaperones from cytosol and from organelles were present, because a considerable breakage of plastids probably occurs during the mechanical disruption of the plant tissues. To the contrary, intact chloroplasts were isolated through a Percoll gradient, a method that had been shown to avoid contamination of chloroplasts with cytosolic factors involved in protein import (40). Thus, the stroma obtained from them did not contain cytosolic or envelope factors and will mainly have stromal Hsp70 molecular chaperones. We incubated GST-TP fusion proteins with whole cellular extracts from pea leaves or chloroplast stroma at identical final protein concentration. Then, complexes between GST-TPs and the interacting proteins from the extracts were purified, washed, resolved in SDS-PAGE, and analyzed by Coomassie Brilliant Blue staining, or Western blotting using two different antibodies. One of them (anti-Hsp70) is a monoclonal antibody raised against human Hsp70 that also reacts against plant Hsp70s. The other is a polyclonal antibody prepared against purified recombinant CSS1, a chloroplast Hsp70 also known as Hsp78. As shown in Fig. 2A (lanes 1 and 4), the human monoclonal antibody reacted with the Hsp70s present in whole cellular extracts but did not recognize Hsp70 molecular chaperones present in purified chloroplast stroma. To the contrary, anti-CSS1 detected a chloroplast chaperone present in stroma and whole cellular extracts (Fig. 2B, lanes 1 and 4). When the isolated complexes were examined, we observed that the protein of ~70 kDa, which always co-purified

### Table 1

| Precursor | Transit peptide |
|-----------|-----------------|
| preFNR    | MAAAATAVSEPYSTSTSPRTSIVAPERHKKVSIINVSISGRVTEQYTTEA... |
| preFNR-14 |                 |
| preFNR-23 |                 |
| preFNR-1234 |            |

*Fig. 1. Primary sequences of the transit peptides and expression and purification of the wild type and mutant preFNR precursors. A, amino acidic sequences of wild type and mutant preFNRs transit peptides showing the amino acid substitutions introduced in the mutant versions of the transit peptides (boxes). Numbers indicate the first and last amino acids of transit peptides. B, wild type and mutant preFNRs (preFNR-14, preFNR-23, and preFNR-1234) were expressed in E. coli, purified as fusion proteins to thioredoxin (Trx-preFNRs), and digested with thrombin to separate the precursor from the carrier protein. Purified proteins were analyzed by 10% SDS-PAGE and Coomassie Brilliant Blue staining.*
with GST-TP, was reactive only with anti-CSS1 antibodies (Fig. 2B, lanes 2 and 3). It had been previously reported that the molecular mass of mature CSS1 is about 69 kDa (22). No reaction of the purified samples was observed using antibodies against cytoplasmic Hsp70 (Fig. 2A, lanes 1 and 3). The reactive band in Fig. 2B (lanes 2 and 3) was of identical molecular weight to the one observed when chloroplast stroma was analyzed with the same antibodies (Fig. 2B, lane 1). Identical results were obtained when the stroma used in the incubations was purified from chloroplasts that were previously treated with thermolysin, a protease that does not penetrate the chloroplast outer membrane, thus excluding the possibility that the chaperone bound to the fusion protein was a cytoplasmic contaminant (not shown). When the complexes were analyzed by SDS-PAGE using Coomassie Brilliant Blue staining (Fig. 2C), or Western blotting using monoclonal anti-Hsp70 (Fig. 2D), a band of ∼70 kDa with identical molecular mass to the one observed by Western blotting was observed. A high affinity binding (*K_d* 125 nM) occurs with the wild type transit peptide, meanwhile introduction of the four mutations decreased about seven times the *K_d* (880 nM for the GST-TP-1234-Hsp70 complex).

Then, the effect of the addition of chloroplast Hsp70 during an *in vitro* import assay was investigated. Fig. 3 shows that stromal extracts, or stromal extracts plus purified CSS1 molecular chaperones, reduced the import of preFNR to isolated chloroplasts in 30 and 40%, respectively. Experiments performed with different chloroplast preparations gave essentially the same results, showing an increase of inhibition by the addition of purified CSS1 with respect to the inhibition observed with stroma extracts. The effect was probably due to the binding of the chloroplast chaperones to the transit peptide before import. When the preFNR with all the Hsp70 binding sites modified in its transit peptide was used for the import reaction, no effects were observed either by the addition of stroma, or by the stromal extracts plus purified CSS1 molecular chaperones (Fig. 3). Then, we hypothesized that if a stromal Hsp70 acts as the main motor during translocation, weakening its interaction with transit peptides should impair polypeptide import. Therefore, we studied the binding and import to isolated chloroplasts of the wild type, and mutant preFNRs.

**Binding of Wild Type and Mutant Precursors to Chloroplast**—Fig. 4 shows the binding of the wild type preFNR and the preFNR-1234 to isolated intact chloroplasts. Organelles were pretreated by incubation in the dark at 25 °C during 15 min. Then, chloroplast suspensions were incubated with different concentrations of precursors in the presence of 50 μM MgATP, which is known to promote the formation of the early import intermediate, but does not sustain protein import. The
Import of Wild Type and Mutant Precursors to Chloroplasts—In vitro precursor import experiments to isolated pea chloroplasts were performed with the wild type preFNR and the preFNR-1234 variant under identical conditions. A typical time course experiment is shown in Fig. 5A. A steady linear rate during the first 2 min of reaction was obtained under the experimental conditions used. These rates were taken as initial activities and always used for kinetics parameters calculation (inset in Fig. 5B). As can be observed in Fig. 5B, the import rates did not increase linearly after 2 min of reaction and leveled off for both precursors. However, the import reactions continued up to 20 min. Differences in import rates were evident at the beginning of the import reaction and then remained constant after 5 min of reaction (Fig. 5B). Initial activities obtained with different concentrations of precursors were adjusted using a Michaelis-Menten rate equation, and average $K_m$ and $V_{max}$ values were estimated (Fig. 6). As shown in Table I, import of the wild type preFNR displayed $K_m$ and $V_{max}$ values of about 406 ± 194 nM and 10,195 ± 1,888 molecules of precursors imported per minute by plastid, respectively. These values are statistically significant and reasonably coincident with kinetics parameters previously obtained for this process (41–43). In each set of experiments, the wild type and the mutant precursors were analyzed in parallel using the same chloroplast preparation. Mutated precursors were imported in all cases with apparent $K_m$ similar to the one obtained for the wild type precursor, but with higher $V_{max}$. For the preFNR-
1234 a $V_{\text{max}}$ of 19,989 $\pm$ 1,476 was obtained. Values obtained for precursors containing two mutations instead of four also show an increase of $V_{\text{max}}$, but of minor extent than the one with four mutations (Fig. 6 and Table I). The effect of these mutations on the protein import process were shown to be non-accumulative as in the case of the alteration of the in vitro release of bound *E. coli* Hsp70 molecular chaperones to the transit peptide (21).

We then analyzed the import of wild type and mutated preFNRs as a function of precursor concentration in in vitro assays during 20 min, varying the concentrations of precursor from 70 to 2800 nM (Fig. 7). Even though import rates were not linear in these conditions, we attempted to exert the system to its maximum import capabilities. We observed that preFNR-1234 was imported to a greater extent than the wild type precursor, and this difference was higher at concentration above 500 nM. It is worth noting that, at 2800 nM, a rather high precursor concentration, preFNR-1234 was imported 50% more than the wild type at the same concentration. For the wild type precursor, only a subtle augment of the number of molecules imported by chloroplast was observed at concentrations higher than 1000 nM. To the contrary, preFNR-1234 import increased steadily above 1000 nM, with an increment of 32% from 1000 to 2800 nM (Fig. 7). As a control, chloroplasts were re-isolated after import, kept at 25 °C for 15 min, and compared with chloroplasts analyzed immediately after import and re-isolation. No decay was observed in the number of imported proteins for the wild type and the mutated precursors (not
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Fig. 6. Determination of the import rate constants for the different preFNR variants. Import assays as a function of precursor concentration were performed in the light at 25 °C during 2 min. Samples were analyzed by SDS-PAGE, electrotransferred to nitrocellulose, and autoradiographed. Error bars give the deviation of three repeat measurements. PreFNR (△), preFNR-14 (●), preFNR-23 (▲), and preFNR-1234 (●).

Fig. 7. Import of preFNR and preFNR-1234 variants into isolated pea chloroplasts. Import assays as a function of precursor concentration were carried out during 20 min in the light at 25 °C. Samples were analyzed by SDS-PAGE, electrotransferred to nitrocellulose, and autoradiographed. Error bars give the deviation of three repeat measurements. PreFNR (△) and preFNR-1234 (▲). Values on the y-axis represent the number of precursor molecules imported by chloroplast in 20 min at each stated concentration.

shown), indicating that differences depicted in Fig. 7 were not the result of proteolytic degradation of the imported proteins.

We have also performed import experiments using urea-denatured wild type precursor and preFNR-1234 to avoid any restriction imposed by the folding of the mature part of the preprotein. The amount of precursors imported in 0.5- and 20-min reactions was determined. Both precursors showed an increment in the number of imported molecules by intact pea chloroplasts. Import experiments were performed during 0.5 and 20.0 min and represented as the number of precursor molecules imported by chloroplast during these periods of time. Numbers above the brackets indicate the increase observed in import rates for the mutated precursor with respect to the wild type form, both folded, or previously unfolded with urea. All import reactions were carried out in the light at 25 °C. Samples were analyzed by SDS-PAGE, electrotransferred to nitrocellulose, and autoradiographed. Error bars give the deviation of three repeat measurements.

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The import of unfolded preFNR and preFNR-1234. Precursors were unfolded with 8 M urea before their incubation with intact pea chloroplasts. Import experiments were performed during 0.5 and 20.0 min and represented as the number of precursor molecules imported by chloroplast during these periods of time. Numbers above the brackets indicate the increase observed in import rates for the mutated precursor with respect to the wild type form, both folded, or previously unfolded with urea. All import reactions were carried out in the light at 25 °C. Samples were analyzed by SDS-PAGE, electrotransferred to nitrocellulose, and autoradiographed. Error bars give the deviation of three repeat measurements.

Fig. 8. Import of unfolded preFNR and preFNR-1234. Precursors were unfolded with 8 M urea before their incubation with intact pea chloroplasts. Import experiments were performed during 0.5 and 20.0 min and represented as the number of precursor molecules imported by chloroplast during these periods of time. Numbers above the brackets indicate the increase observed in import rates for the mutated precursor with respect to the wild type form, both folded, or previously unfolded with urea. All import reactions were carried out in the light at 25 °C. Samples were analyzed by SDS-PAGE, electrotransferred to nitrocellulose, and autoradiographed. Error bars give the deviation of three repeat measurements.

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gel bands, providing a comparative analysis of their structural stability (34). Two parameters were obtained from the gels patterns: C̅m, which represents the urea concentration at the transition midpoint, and m, calculated as the slope of ΔG versus urea concentration at C̅m (m = −4RT/ΔC̅m) (45). All precursor proteins showed continuous unfolding transitions with similar m values and negligible changes in C̅m with a concentration media of 8 M urea (Fig. 9 and Table I), as described previously for the mature FNR (33) and preFNR (29) (Table I). Consequently, it can be concluded that no significant changes in thermodynamic stability of the proteins were produced by the mutations.

**DISCUSSION**

In this study we have challenged the participation of stromal Hsp70s as the main driving force during chloroplast protein import. Our results indicate that mutant preFNR precursors, which show altered interaction with Hsp70 molecular chaperones in their transit peptides, are imported more efficiently than the wild type precursor. The interactions of transit peptides with molecular chaperones have been extensively studied. In mitochondria several cytosolic factors have been proposed to influence the folding and protein import, and they are widely documented (46). Hsp70 molecular chaperones from cytosol and mitochondrial matrix interact with both presequences and mature parts of precursors (19, 20, 47, 48). Standing evidences correlate the mitochondrial Hsp70s located in the matrix with the mitochondria protein import (49–51). Similarly, it has been pointed out and experimentally proved that the Hsp70 from endoplasmic reticulum acts as a molecular ratchet during post-translational protein transport (52).

Here we show that the transit peptide of preFNR interacts preferentially with stromal Hsp70 molecular chaperones, and under the same conditions, no interaction with the plant cytosolic molecular chaperones was observed. Precursor proteins
containing a specific phosphorylation motif on their transit peptides interact with 14-3-3 proteins and cytosolic Hsp70 molecular chaperones after their phosphorylation (53). No predicted 14-3-3 binding motifs were observed on the preFNR transit peptide. However, at present we cannot establish if preFNR belongs to the above mentioned type of precursors or to a group of preproteins that follows a different guiding mechanism to reach the organelle surface. It was recently shown that a soluble factor from wheat germ lysate interacts directly with the precursor protein and renders it import-incompetent. The proteinaceous factor does not affect the import of precursors already bound to chloroplast (54). The authors assert that pea leaf extracts may also contain such factor. When we assayed the effect of stromal Hsp70 on the import of the mutant preFNR-1234, no inhibition was observed, in agreement with the reduced interaction detected in vitro between the mutant transit peptide and the stromal Hsp70 CSS1 (Fig. 2, C and D). Moreover, addition of purified CSS1 during import assays promoted higher inhibition of the wild type precursor translocation. Both observations corroborate that the interaction among the wild type precursor protein and CSS1 was responsible for the detected inhibitory effect. These results indicate that the inner force that drives the polypeptide import may not be related to the interaction that had been abolished in the preFNR-1234. Further investigation of the import of the mutant precursors showed that introduction of mutations on the transit peptide that decreased stromal Hsp70 binding increased the $V_{\text{max}}$ of protein import. When the four putative Hsp70 binding sites were modified, an increase of about 50% in import rates was observed. The significant $V_{\text{max}}$ increase was only observed for the precursor protein carrying the four mutations, whereas the double mutants only showed a slight increase of $V_{\text{max}}$. The non-additive behavior of the mutations on increasing the efficiencies of import may be related to the vectorial character of the process. Moreover, not all mutated sites should necessarily be equivalents for the import process, as shown in our experiments.

Recently, it has been experimentally sustained that the overall context, and not a specific amino acidic sequence of the transit peptide is relevant for the import efficiency (55). The increase that is observed in the import rates of our mutated precursor may be directly related to the fact that, from the four introduced mutations, three of them replaced amino acids with large R groups by smaller ones (Leu to Ala and Ile to Met). Experiments devised to examine the polypeptide diffusion in the mitochondrial translocation pore showed that precursor chains passively interact with the channel (56). Chauwin et al. (39) mathematically evaluating these data concluded that the polypeptide is not free but is hindered by strong interactions with the wall of the pore. Moreover, they had concluded that, in the case of mitochondria, mHsp70 should be an efficient motor comparable to myosin or kinesin to overcome the drag forces in the pore during the translocation of a polypeptide. In chloroplasts, Hinnah et al. (57) have recently analyzed the properties of Toc75, the protein import pore of the outer chloroplast membrane. These authors concluded that the pore has a wider vestibule and a central constriction zone of only 14 Å. Thus, although the pore may be a flexible structure able to expand during polypeptide import as suggested, modification of the volume of the peptide being translocated may have a considerable influence on its import rates. Because no differences in the enzymatic activity and the overall stability of the mutated precursors were detected (see Table I), the observed changes in import rates should be attributed solely to the interaction of the transit peptide with one or more members of the import machinery. Consistently, although both wild type and mutant unfolded precursors exhibited increased import rates with respect to their folded forms, this increment was more important for the wild type preFNR in the first 0.5 min of import reactions. These observations suggest that folding is imposing restrictions to the polypeptide translocation and that the import machinery more efficiently overcomes these restrictions when the mutated precursor is the one being translocated.

An important aspect of the protein import process should be considered. The participation of a ratchet or power stroke mechanism became particularly relevant when the precursor protein is tightly folded, as in the case of preFNR. We previously observed that this precursor remains folded when bound to chloroplast, and probably the polypeptide unfolding takes place during translocation itself (28). In this case, the import machinery has to exceed the pore-dragging forces plus the unfolding of the polypeptide. Gaume et al. (14) determined that mitochondrial presequences longer than 52 amino acids were able to span both membranes and tightly interact with matrix mitochondrial Hsp70. We have observed that in the crystal structure of a FNR mutant (Protein Data Bank entry 1QGA (58)), which is near equivalent to the wild type FNR, the first 14 amino acids of the mature protein are completely exposed out of the folded structure. Therefore, the sum of the transit peptide plus the exposed N-terminal region of the mature part of the protein totalized 66 amino acids. Thus, if an internal Hsp70 protein was responsible for the unfolding and the inward movement of the polypeptide being imported, the main translocational force should be applied within the transit peptide, the only available structure emerging from the inside face of the envelope. Our results clearly show that a transit peptide with reduced capability to interact with the internal Hsp70 molecular chaperone CSS1 has the ability to drive protein import at higher velocity and efficiency. Accordingly, we had previously observed that precursors with deletions in the 14 amino acids of the N-terminal region of the mature protein showed increased import rates compared with the wild type (29). We are not precluding that the interaction between chloroplast Hsp70 molecular chaperones and the mature part of the precursor proteins may help or drive the import process. However, these Hsp70 binding sites will be accessible only after the import machinery unfolds the polypeptide through its N termi-
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