The import of proteins into chloroplasts involves a cleavable, N-terminal targeting sequence known as the transit peptide. Although the transit peptide is both necessary and sufficient to direct precursor import into chloroplasts, the mature domain of some precursors has been shown to modulate targeting and translocation efficiency. To test the influence of the mature domain of the small subunit of Rubisco during import in vitro, the precursor (prSSU), the mature domain (mSSU), the transit peptide (SS-tp), and three C-terminal deletion mutants (Δ52, Δ67, and Δ74) of prSSU were expressed and purified from Escherichia coli. Activity was then evaluated by competitive import of 35S-prSSU. Both IC50 and Kᵢ values consistently suggest that removal of C-terminal prSSU sequences inhibits its interaction with the translocation apparatus. Non-competitive import studies demonstrated that prSSU and Δ52 were properly processed and accumulated within the chloroplast, whereas Δ67 and Δ74 were rapidly degraded via a plastid-localized protease. The ability of prSSU-derived proteins to induce inactivation of the protein-import-related anion channel was also evaluated. Although the C-terminal deletion mutants were less effective at inducing channel closure upon import, they did not effect the mean duration of channel closure. Possible mechanisms by which C-terminal residues of prSSU modulate chloroplast targeting are discussed.

Many chloroplast proteins are nuclear-encoded and produced in the cytosol of the plant cell. Nuclear-encoded chloroplast proteins are predominantly synthesized as larger molecular weight precursors containing an N-terminal transit peptide. The transit peptide (tp) is a cleavable N-terminal signal sequence that mediates precursor binding and import into the chloroplast via an ATP-dependent mechanism. Following transport of the precursor through the chloroplast envelope, the transit peptide is cleaved by the stroma-processing peptidase to generate the mature protein. Transport of precursors across the chloroplast envelope has been shown to involve distinct protein complexes within the outer membrane (Toc, translocation apparatus of the outer membrane of chloroplasts) and inner membrane (Tic, translocation apparatus of the inner membrane of chloroplasts) (1–4). These two complexes are believed to interact at inter-membrane contact sites, which enables precursor translocation in a single coordinated step (5, 6).

Recently, an inner membrane localized anion channel, PIRAC (Protein Import Related Anion Channel), has been shown to be involved in chloroplast protein import. PIRAC is a 50-picoSiemen anion channel that becomes inactivated during chloroplast protein transport via a mechanism that requires both a functional transit peptide and a stromal ATP source (7). The exact role of PIRAC in protein import into the chloroplast is not defined as yet, however, at least three different mechanisms may explain channel inactivation: (i) PIRAC may function as the protein import channel in the inner membrane of chloroplasts, and inactivation represents a shift from ion conductance to protein conductance; (ii) PIRAC may be intimately associated with translocation components of the inner membrane such that a precursor-induced conformational change results in inactivation of the ion channel; or (iii) the formation of contact sites between Toc and Tic during translocation causes transient inactivation of the ion channel. Further investigation is needed to elucidate the precise role of PIRAC in protein import into the chloroplast.

Current models suggest that transit peptides are both necessary and sufficient to direct the targeting and import of precursor proteins into the chloroplast. However, direct experimental evidence is not entirely consistent with these models. For example, the attachment of transit peptides to unrelated precursors confers proper targeting to some but not all proteins (8–11). In addition, no common motif has been identified among the several hundred transit peptides that have been sequenced (12). To explain this apparent enigma, it has been postulated that a common secondary or tertiary structure involving the mature domain might provide both targeting specificity and translocation efficiency (6). Consistent with this notion, chimeras constructed between the transit peptide and various portions of the mature domain have been shown to improve import efficiency (8, 10). Also, deletion of C-terminal sequences from various precursors has been shown to interfere with one or more steps of the translocation pathway, depending on the precursor studied and the extent of the C-terminal deletion (11). These studies collectively suggest that contribu-
tions from the mature domain play an important role in facilitating the interaction of transit peptides with the translocation apparatus. However, a more systematic and quantitative study is needed to identify mature domain sequences that directly influence chloroplast import.

In this work, we have evaluated the import activity of the precursor to the small subunit of Rubisco (prSSU), the mature domain (mSSU), the full-length transit peptide, (SS-tp), and three prSSU mutants that lack 52, 67, or 74 C-terminal amino acids, respectively. Using these proteins as competitive inhibitors of the radiolabeled precursor during import, we demonstrated that the transit peptide is absolutely required for interaction with the translocation apparatus. However, a specific region within the mature domain strongly influences this interaction. In addition, we demonstrated that sequences within the C terminus of prSSU also influence the interaction between the transit peptide and PIRAC. The findings indicate that the C terminus of prSSU plays an important role in protein translocation.

MATERIALS AND METHODS

Plant Growth and Chloroplast Isolation—Dwarf pea seedlings (Pisum sativum, Laxton Progress number 9) were grown, and chloroplasts isolated as described by Bruce et al. (13). Chloroplasts for electrophysiology measurements were isolated from pea leaves by cutting with a razor blade in 2.5 mM Tes/KOH, pH 7.2, 225 mM sorbitol, 25 mM KCl, and 2 mM CaSO₄. This preparation was transferred directly to a 2-ml chamber, which was mounted on a light microscope to allow visual selection of single intact chloroplasts.

Generation of C-terminal Deletions—The full-length precursor was isolated from pET11-prSSU (14) using BamHI and NcoI, and subcloned into the same sites of pET23d (Novagen). Exonuclease III digestion was performed using the Erase-a-Base kit (Promega). To determine the extent of the deletions, direct colony polymerase chain reaction was performed using Tₜ promoter primers. Plasmid DNA was isolated and sequenced in both directions using an ABI automated sequencer. Three deletion mutants, prSSΔ52 (Δ52), prSSΔ67 (Δ67), and prSSΔ74 (Δ74) were generated that lacked 52, 67, and 74 amino acids, respectively, at the C terminus. The Δ74 deletion contained an additional 6 amino acids (ATVVRM) at the C terminus due to read through into vector sequence. All other regions of the precursor were unchanged.

Preparation of Precursor Proteins and the Transit Peptide—Recombinant proteins were expressed and isolated from the BL21(DE3) strain of Escherichia coli as described by Pinnaudawg and Bruce (15). The final inclusion body pellet was then solubilized in 8 M urea and 50 mM dithiothreitol. SS-tp was expressed and purified from the BL21 strain of E. coli as described by Pinnaudawg and Bruce (14).

In Vivo Radiolabeling of prSSU and Deletions—prSSU and prSSU mutants were radiolabeled in vivo radiolabeling experiments. The cells were grown for 8 h in Dulbecco’s modified Eagle’s medium (BioWhittaker) without cysteine and methionine, followed by incubation with Tran35S-Label metabolic labeling reagent (ICN). The inclusion bodies were isolated using BugBuster™ protein extraction reagent (Novagen) and solubilized in 8 M urea + 50 mM dithiothreitol. Specific activity was 1–5 × 10⁵ dpm/μg protein.

In Vitro Protein Import Assays—Kinetin import assays were performed by incubating increasing concentrations of 35S-labeled prSSU with freshly prepared chloroplasts (50 μg of chlorophyll) in the presence of 1× Import Buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 8.0) containing 5 mM Mg-ATP, 10 mM dithiothreitol. Urea concentration was kept constant at 300 mM and was shown not to affect import (data not shown). Addition of 35S-prSSU started the assay. After 10 min at room temperature, the assay was terminated by rapid dilution in ice-cold 1× Import Buffer (5-fold) and immediately placing the samples on ice in the dark. Intact plastids were re-isolated according to Bruce et al. (13) and were prepared for SDS-PAGE.

In Vitro Protein Import Competition Assays—Competition import assays were performed by incubating 100 nM 35S-prSSU and increasing concentrations of competitor protein with freshly prepared chloroplasts as described above. Competitions proceeded for 15 min at room temperature. To start the assay, 35S-prSSU and the competitor protein were added in rapid succession (<3 s between additions) (15). Import assays were performed as described above.

Time Course of Import and Degradation—Time course import assays of 35S-precursors were performed using 300 nM of each precursor as described above. Assays were performed at room temperature in the dark. Addition of the 35S-precursor started the assay, and 100-μl samples were removed at timed intervals, diluted 10-fold with ice-cold 1× Import Buffer, and placed on ice in the dark. Intact chloroplasts were re-isolated from each sample as described by Bruce et al. (13). These samples represent the re-purified chloroplast sample. Also at each time interval, 50 μl of the import reaction was removed and immediately mixed with 50 μl of 2× SB and boiled for 3 min. These samples represent the total import reaction.

Data Analysis—Data analysis was performed by electronic, filmless autoradiography (Instant-Imager, Packard Instruments), and quantification of signal by InstantImager analysis software (Packard Instruments). Signals were reported as counts per minute (cpm) and converted to molecules of mSSU/chloroplast/min using the counting efficiency of the instrument, the specific activity of the precursor, the cysteines and methionines per molecule, the number of chloroplasts per ml of import reaction, and the length import time. The data were analyzed using GraphPad Prism™ (GraphPad Software, Inc.) computer software for enzyme kinetics and the Kᵢ and Vₘₐₓ determined using nonlinear regression. These calculations were performed unless otherwise noted.

 Dixon Analysis—For Dixon analysis, competition import reactions were performed as described above with either 60 nM or 180 nM 35S-precursor plus increasing concentrations of cold competitor. Rates were calculated at 200, 500, 1000 nM of each cold competitor. ATP was added to the bath solution (stoma equivalent) to 0.5 mM for ~5 min before electrophysiological experiments were started.

Currents were measured with an Axopatch 200B patch clamp amplifier (Axon Instruments). The data were filtered at a cut-off frequency of 1 kHz, using an 8-pole Bessel filter (internal filter of the Axopatch 200B). The data were filtered digitized at 10 kHz using aCED 1401+ and analyzed with the Patch and Voltage Clamp software (Cambridge Electronic Design) (16). Potentials are given with regard to the pipette interior, the bath was kept at ground, using a 250 mM KCl agar bridge. To determine the distributions of open and closed time durations of PIRAC, a module was developed in the matrix-calculating software Matlab (Mathworks, Inc.). This module uses the 50% threshold method to identify transitions of the channel between the open and the closed state. The open distributions were fitted with a Gaussian density functions using the maximum likelihood method (17).

Protein Measurements—Purified proteins were quantified by commercial Bradford reagent (Bio-Rad). Quantification of total chloroplast protein was performed using the BCA protein quantification assay (Pierce).

RESULTS

Generation and Purification of C-terminal Deletions—To determine the effect of prSSU C-terminal sequences in chloroplast protein import, serial C-terminal deletions were generated and characterized. Out of the several hundred clones originally generated, only a small subset was characterized further, based on deletion length and ease of purification from inclusion bodies when expressed in E. coli (data not shown). The location and size of these deletions are shown in Fig. 1. Variants of prSSU that lack 52, 67, and 74 amino acids at the C terminus were denoted Δ52, Δ67, and Δ74. We were unable to detect a deletion that yielded an insoluble protein shorter in length than the Δ74 deletion, suggesting that sequences within this region are directly involved in inclusion body formation. The purification of prSSU, mSSU, GST-tp, and SS-tp was reported previously (15). The Δ52, Δ67, and Δ74 deletions were isolated by a similar method and analyzed for purity by SDS-PAGE.
FIG. 1. Comparison of mSSU, prSSU, GST-tp, SS-tp, and C-terminal deletions. The position and length of the transit peptide and mature domain has been drawn to scale. The region corresponding to the full-length transit peptide is shown in gray, and the region corresponding to the mature domain is shown in black. The small white block between GST and SS-tp represents two amino acids (GS) introduced during subcloning. Glutathione S-transferase protein is shown with the diagonal pattern and has been condensed for space constraints. The amino acid sequences for prSSU, mSSU, and the deletions (prSSΔ52, prSSΔ67, and prSSΔ74) were obtained from DNA sequence analysis of the cloned genes. The actual molecular weight is shown in Daltons on the right.

FIG. 2. Purification of E. coli expressed prSSU, mSSU, Δ52, Δ67, Δ74, GST-tp, and SS-tp. Overexpressed prSSU, mSSU, Δ52, Δ67, and Δ74 were purified to near homogeneity. SDS-PAGE and Coomassie Brilliant Blue stain of purified precursors (5 μg of total protein loaded). Purified protein loaded in each lane is shown at the top of the figure.

Purified prSSU, mSSU, GST-tp, and SS-tp were used as competitive inhibitors of 35S-prSSU import in vitro. The concentration of prSSU used in each import reaction is shown at the top of the gel. Import reactions were conducted as described under “Materials and Methods.” Chloroplast loading was normalized by BCA protein measurements. E, quantitative analyses of import data using increasing concentrations of 35S-prSSU. The inset shows the data represented as an Eadie-Scatchard analysis. Data are presented as mean ± S.D.

FIG. 3. In vitro import kinetics. A, electronic autoradiogram of a representative import reaction as a function of increasing concentrations of 35S-prSSU. The concentration of prSSU used in each import reaction is shown at the top of the gel. Import reactions were conducted as described under “Materials and Methods.” Chloroplast loading was normalized by BCA protein measurements. E, quantitative analyses of import data using increasing concentrations of 35S-prSSU. The inset shows the data represented as an Eadie-Scatchard analysis. Data are presented as mean ± S.D.

In Vitro Import Kinetics—To define the kinetic parameters of prSSU import in vitro, the rate of import (processing to mSSU) as a function of prSSU concentration was determined. The amount of 35S-prSSU substrate was varied from 10 nM to 1.6 μM in a 10-min in vitro import assay (Fig. 3A). This time point was within the linear range of the time course of import (data not shown). Three independent import assays were performed and the amount of mSSU product was directly quantified (Fig. 3B). The average product formed at each substrate concentration was directly quantified (Fig. 3C). The capacity of the translocation apparatus was determined by calculating the import efficiency from the calculated molecular masses of 13.2, 12.5, and 11.6 kDa, respectively, for the deletions. We have no explanation for the abnormal electrophoretic mobilities.

Import Competitions and IC50 Determination of C-terminal Deletions—To identify the regions within the mature domain of prSSU that facilitate import, prSSU C-terminal deletions (Δ52, Δ67, and Δ74) were used as competitive inhibitors in 35S-prSSU import assays. All three deletions were tested with the same preparation of chloroplasts to minimize experimental variability. Decreasing levels of mSSU accumulation with increasing competitor concentration indicated that all three pre-
cursor mutants were able to compete with \(^{35}\text{S}\)-prSSU for import into the chloroplast (Fig. 5A). However, the longer precursor mutants competed more effectively than the shorter mutants. Quantification of three independent experiments (Fig. 5B) resulted in IC\(_{50}\) calculations of 33 ± 15.3, 145 ± 26.9, and 305 ± 40.8 nM for the \(\Delta 52\), \(\Delta 67\), and \(\Delta 74\) mutants, respectively. Plotting these values as a function of mature domain length illustrates the relationship between precursor length and activity as a competitive inhibitor (Fig. 6). Although removal of the last 52 amino acids from the C terminus of prSSU only slightly changes the affinity of the precursor for the translocation apparatus, removal of an additional 15 amino acids increases the IC\(_{50}\) from 33 ± 15.3 nM to 145 ± 26.9 nM and removal of an additional seven amino acids increases the IC\(_{50}\) further to 305 ± 40.8 nM. These findings suggest that sequences between the C termini of the \(\Delta 52\) and \(\Delta 74\) mutants contribute significantly to the process of chloroplast protein targeting/translocation.

**Dixon Analysis of IC\(_{50}\) Values for prSSU and C-terminal Deletions**—To determine the inhibition constant, \(K_i\), for prSSU and the three C-terminal deletion mutants, Dixon analysis was performed using \(^{35}\text{S}\)-prSSU as substrate and unlabeled prSSU, \(\Delta 52\), \(\Delta 67\), or \(\Delta 74\) as competitive inhibitors of import. \(^{35}\text{S}\)-prSSU was fixed at 60 and 180 nM, and the inhibitor concentration was varied from 50 nM to 1.2 \(\mu\)M (Fig. 7A). Data were fit by linear regression (Fig. 7B) and the intersection point of the lines is defined as \(-K_i\). The \(K_i\) values for prSSU (105 nM), \(\Delta 52\) (66 nM), \(\Delta 67\) (110 nM), and \(\Delta 74\) (437 nM) are consistent with the IC\(_{50}\) values for each competitor (Table I). Successive deletion of sequences within the prSSU mature domain results in less effective import inhibition, suggesting that an element within...
the mature domain modulates targeting and/or translocation efficiency.

Attenuated import competition by the prSSU deletion mutants could result from disrupted binding at the chloroplast surface, impaired translocation through Toc and Tic, or some combination of these processes. To explore a binding-specific effect, binding studies were performed with prSSU and the precursor mutants at low levels of ATP (data not shown). The binding results demonstrated that removal of 52, 67, and 74 amino acids from the C terminus did not significantly affect binding of these precursors to the chloroplast presumably via Toc components.

Import Time Course of prSSU and C-terminal Deletions—Except for mSSU and GST-tp, all of the proteins tested have been shown to compete for the import of $^{35}$S-prSSU. To directly determine whether the C-terminal deletion mutants of prSSU are competent for chloroplast import in vitro, the truncated precursors were metabolically labeled with $^{35}$S and used as substrates in import time course experiments. Monitoring the level of $^{35}$S-labeled protein remaining in both the reaction mixture and within re-isolated chloroplasts permits direct quantification of the substrate remaining at each time point, as well as the amount of imported/processed mature form. In this way, both the import rate and the extent of mSSU accumulation for each precursor may be determined and directly compared.

The four autoradiogram panels on the left of Fig. 8A indicate the precursor pool size remaining in each import reaction as a function of import time. Quantification of these data are shown in Fig. 8B, where the amount of precursor was normalized to the 1 min time point allowing comparison between the different precursors. All four proteins, prSSU, Δ52, Δ67, and Δ74, were imported at a similar rate, as seen in Fig. 8A and quantified in Fig. 8B. Re-isolation of the chloroplasts, however, demonstrated that only full-length prSSU and the Δ52 precursor imported and accumulated as mature processed proteins. Although the level of Δ67 and Δ74 truncates precursors clearly decreased as a function of import time, these mutants failed to accumulate to detectable levels as a processed form in the stroma. Taken together, these results indicate that all four precursor proteins import successfully into the chloroplast but that the Δ67 and Δ74 truncated precursors are subject to rapid degradation following import. Neither the identity nor the sub-organelar location of the protease responsible for this degradation is known.

Interaction of prSSU and C-terminal Deletions with PIRAC—Thus far, we have demonstrated that sequences within the mature domain of prSSU enhance the efficiency of transit peptide-mediated import into chloroplasts. These findings implicate an interaction of the precursor with transport complexes, Toc and Tic. However, the anion channel, PIRAC, is also involved in chloroplast protein import since a functional transit peptide and full-length precursor have been shown to inactivate the channel (18). To evaluate whether precursor interaction with PIRAC is also modulated by sequences in the mature domain, single channel recordings of PIRAC conductance were measured in the presence of prSSU, mSSU, and the prSSU C-terminal deletion mutants. Fig. 9A shows the single channel recordings of PIRAC conductance for a control excised patch and for an excised patch after protein addition. Fig. 9B represents the all points amplitude histogram of these recordings. Addition of prSSU to the envelope patch decreases the open probability ($P_O$) of the channel from 0.81 ± 0.04 in the control situation to 0.17 ± 0.08, indicating that prSSU induces channel inactivation. In contrast, addition of mSSU induced no significant change in PIRAC activity, as indicated directly in Fig. 9A, and in the failure of this protein to decrease the $P_O$ measured in the all points amplitude histogram in Fig. 9B. The deletion mutants of prSSU, however, induced PIRAC inactivation in a manner that was proportional to the length of the mature domain of the precursor. The $P_O$ of PIRAC in the presence of Δ52, Δ67, and Δ74 were 0.45 ± 0.07, 0.53 ± 0.07, and 0.71 ± 0.07, respectively (Table II). These data indicate that the transit peptide is necessary for PIRAC inactivation, as was shown previously (7, 18), and that the removal of the C terminus of the precursor attenuates this interaction.

Under control conditions (i.e. in the absence of any precursor) and in the presence of mSSU, three closed channel states with characteristic mean durations of 0.35 ± 0.03, 2.41 ± 0.98, and 29.1 ± 6.8 ms and two open channel states with mean durations of 0.88 ± 0.34 and 47.2 ± 9.6 ms have been detected for PIRAC (Table II) (18). However, in the presence of prSSU, an
additional closed state ($\tau_{AC}$) with a much longer duration was detected (18). The duration of this new long-lived closed state of PIRAC is $989 \pm 214$ ms. All of the truncated precursors induced a similar long-lived closed state with a mean duration of $\approx 1300 \pm 400$ ms (Table II). The observation that full-length prSSU and the deletion mutants decreased the $P_D$ of PIRAC, yet did not significantly change the duration of $\tau_{AC}$, suggests that these precursors interact with PIRAC via a common mechanism but that their affinity for the channel is proportional to the length of the mature domain. Whether the difference in affinities results from an attenuation of direct precursor interaction with PIRAC or, alternatively, is a secondary result of a diminished interaction with components of either Toc and/or Tic will require additional study.

**DISCUSSION**

**Kinetic Analysis of prSSU**—The precursor for the small subunit of Rubisco is one of the most well studied precursors, yet kinetic analysis of its in vitro import has not been reported previously. Compared with the few other precursors whose import kinetic parameters have been determined experimentally (prLHCP, prFd, prOEE23, and prOEE33), the $K_m$ of prSSU import is intermediate between the value reported for prFd (120 nM) and prOEE23 (400 nM). The $V_{max}$ of prSSU import is also comparable to that reported for prLHCP. In contrast, the $V_{max}$ of prSSU import is significantly less than the $V_{max}$ reported for either prFd (~22,000 molecules/chloroplast/min) (19) or prOEE23 (>40,000 molecules/chloroplast/min) (20). These differences may reflect genuine disparities in the rate or efficiency of import for the various precursors, or alternatively, they may represent differential responses to chemical denaturation agents used for solubilization of the precursor. As discussed previously by Pilon et al. (19), however, the rates of translocation measured in vitro are quite close to the rates required in vivo for normal maintenance of chloroplast biogenesis, which lends credibility to the use of in vitro import studies as a means of exploring the mechanism of chloroplast protein transport.

**Effect of GST Fusion on SS-tp Activity**—An interesting and significant finding from our in vitro import studies is that the transit peptide of prSSU failed to function as a competitive...
inhibitor of import when GST was fused to the N terminus of the transit peptide. This finding suggests that addition of a 26.6-kDa folded fusion protein inhibits interaction of the N terminus of the transit peptide with one or more components of the targeting/translocation apparatus. Although the precise mechanism of this apparent interaction is unknown, one possibility is that the committed step in binding/translocation occurs when the N terminus of the precursor traverses both envelope membranes and gains accessibility to the stroma. Once the N terminus of the transit peptide stretches into the stroma, binding by one or more molecular chaperones may prevent escape and provide a mechanism for unidirectional ATP-dependent translocation. Evidence from our laboratory indicates that chloroplast transit peptides function in vitro as substrates for hsp70 (21), and that >80% of stromal-targeted precursors in the CHLPEP database contain high affinity hsp70 binding sites at their N termini (21, 22). If this interaction occurs in vivo, then binding by hsp70 in either the stroma (23) or the intermembrane space (3) would require a free transit peptide N terminus, and fusion to GST would predictably block import.

An alternative explanation for the lack of import activity by GST-tp is that the GST domain may interfere with interactions between the transit peptide and lipid components of the chloroplast envelope. Two independent laboratories have shown that the N terminus of transit peptides are capable of interacting directly with chloroplast-specific lipids (15, 24). The GST-tp fusion protein used in this study was shown previously to inhibit the interaction of SS-tp with liposomes whose composition mimics the outer envelope (15). Regardless of the molecular mechanism, however, our findings clearly suggest that accessibility of the N terminus of SS-tp is required for interaction with one or more components of the chloroplast translocation apparatus. Several investigations into the role of different domains of chloroplast transit peptides have also shown that the N terminus is required for precursor import in vitro (24, 25), in vivo (26, 27), and for directing the import of certain fusion proteins in vitro (8). An intriguing aspect of these reports is that N terminus of some transit peptides is often the least conserved region of the entire presequence (26).

**Diminished Activity of SS-tp versus prSSU**—Although popular models contend that the transit peptide is both necessary and sufficient to support the import of chloroplast-destined precursors (6), several reports indicate that the addition of mature domain sequences increases the targeting/translocation activity of certain transit peptides (8–10). We also found that sequences within the mature domain of prSSU enhance import efficiency, only when fused in cis with the transit peptide. The minimal requisite structural elements for engaging the translocation apparatus are contained within the transit peptide, however, not the mature domain. In direct support of this conclusion, short synthetic peptides derived from the SS-tp sequence competitively inhibit prSSU import (28), and similarly, the full-length ferredoxin transit peptide has been shown to import into chloroplasts with no associated passenger protein (29). The full-length precursor was a more effective inhibitor of in vitro chloroplast import, however, and deletion of specific mature domain sequences within the context of prSSU significantly impaired this activity, strongly implicating this mSSU sequence in a beneficial cis interaction with the transit peptide during chloroplast targeting/translocation. The physical/chemical basis for this interaction remains unknown, however.

**Effect of C-terminal Deletions**—Both the IC50 and the Ks values calculated from our experiments indicate that removal of sequences at the C terminus of prSSU decreases the affinity of the protein for one or more components of the translocation apparatus. This effect is not strictly a linear function of precursor length, however, as shown in Fig. 6. Removal of the last 52 amino acids of prSSU may have enhanced import potential, yet removal of a few additional sequences progressively and significantly weakened the ability of the precursors to compete for import. In addition, the Δ52, Δ67, and Δ74 mutations did not affect precursor binding to the chloroplast. Unlike full-length prSSU and the Δ52 mutant, however, the Δ67 and Δ74 precursors did not accumulate in the stroma, suggesting that the decrease in the pool amount may be due to rapid degradation. These observations contrast with the findings of a previous study of prSSU import, which indicate that removal of 26 and 47 amino acids from the C terminus completely abolishes import (11). Even at the earliest point in import time course experiments, the authors did not detect binding or import by the mutant precursors and attributed no import to abolished binding as opposed to rapid degradation. One major difference between that report (11) and our study is that we used purified proteins rather than in vitro translated protein. A potential explanation for the discrepancies in the findings from these two
studies, therefore, is that components in the wheat germ extract may interfere with the import of the mutant precursors.

Stromal Assembly versus Degradation of the Truncated Small Subunit—Direct import of our radiolabeled prSSU mutants into isolated chloroplasts indicated that wild-type prSSU and the Δ52 mutant clearly engaged the translocation apparatus, traversed the chloroplast envelope, and were properly processed into stable appropriately-sized mature forms. In contrast, neither the precursor, mature form, nor discreet degradation products of the radiolabeled precursor were detected. The rate at which all four precursors decreased in the total import reaction was comparable, however, suggesting that each precursor was successfully imported into the chloroplast. Degradation of the Δ67 and Δ74 precursors outside the chloroplast is highly unlikely since the organellar preparations were Percoll-purified and extensively washed. A probable explanation is that the Δ67 and Δ74 mutants were degraded in the stroma following import. Mismodeled proteins, incorrectly translated proteins, nonfunctional proteins, or overproduced subunits are proteolytically degraded in the chloroplast to prevent accumulation of nonfunctional or potentially toxic subunits. Analysis of the Rubisco holoenzyme three-dimensional structure (30) indicates that the deletions in Δ67 and Δ74 remove significant structural elements that intimately contact adjacent large subunits. The Δ52 mutation, however, removes only one unstructured domain that may not be required for folding and/or holoenzyme assembly. The failure of mΔ67 and mΔ74 to assemble properly into the holoenzyme may result, therefore, in rapid degradation via a post-translocational mechanism.

The protease responsible for mΔ67 and mΔ74 degradation is not known, however, a likely candidate is the plastid homologue (ClpC/P) to the bacterial Clp family of serine proteases. ClpC is the regulatory subunit and ClpP the proteolytic subunit. Although both subunits are stromally localized (31), they have also been shown to co-purify with the chloroplast translocation apparatus (32, 33) and were implicated in degradation of mistargeted aberrant prOE33 (34). Further experiments are needed to determine what protease is responsible for proteolytic degradation of mΔ67 and mΔ74.

Effect on PIRAC—Previous studies have demonstrated that PIRAC is inactivated during ATP-dependent protein translocation into the chloroplast (7, 18). This inactivation is reflected by the presence of a precursor-induced long-lived closed state that is absent in the control patches. The fact that PIRAC is localized in the inner envelope and is inactivated by cytosolic localized precursors or transit peptides in the presence of stromal-localized ATP suggests that PIRAC is either a component of Tic or is in some way intimately associated with the Tic complex. All of the precursors and transit peptides tested to date (7, 18) induced a similar long-lived closed state, suggesting that the duration of closure is independent of transit peptide/precursor length. Some other dynamic aspect of the translocon, as opposed to the time needed for completion of polypeptide translocation, may determine the duration of PIRAC closure. The effect of the C-terminal deletions of prSSU on the $P_0$ of PIRAC may result from removing a critical structural element that is required both for precursor interaction with Tic/P and PIRAC, or alternatively, it may be a secondary effect of reduced affinity for a component in the outer envelope, which precedes interaction with PIRAC.

Concluding Remarks—Conflicting evidence in the literature has debated the role of the C terminus of chloroplast precursor proteins in targeting/translocation across the chloroplast envelope. In this first study to directly compare the targeting/translocation activity of a purified precursor, its mature domain, transit peptide, and multiple C-terminal deletion mutants, we have verified a basic tenet of chloroplast import models that the transit peptide is absolutely necessary for import. Our data also demonstrate quantitatively that the interaction of prSSU’s transit peptide with the translocation apparatus is positively modulated by sequences within the mature domain, which function in cis. Moreover, we have partially mapped the regions within the mature domain that are particularly important for this interaction. In addition to facilitating a transit peptide-mediated interaction with components of the translocation apparatus, Tic and P and, sequences within the mature domain of prSSU also contribute to the precursor’s affinity for PIRAC. The structural basis for interactions between disparate regions of the precursor and these distinct chloroplast membrane complexes remains to be determined.

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