Identification of a Translocation Intermediate Occupying Functional Protein Import Sites in the Chloroplastic Envelope Membrane*

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We used complexes of avidin and biotinylated precursors to generate translocation intermediates that occupy functional transport sites and thereby block the transport of other precursor proteins into pea chloroplasts. Cysteine residues of purified precursor to the small subunit of ribulose (prSS) were modified with the biotinylation reagent biotin-1-biotinamido-4-[1'-4']-(maleimidomethyl)-cyclohexane-carboxamido]butane. Chemically biotinylated prSS was readily imported into chloroplasts. The addition of avidin, however, resulted in the formation of an avidin-biotinylated precursor complex that could not be imported into chloroplasts even when precursors had already engaged the transport apparatus before avidin was added. On fractionation, the avidin-biotinylated precursor complex associated with envelope membranes. Titration of transport sites with avidin-biotinylated precursor complexes revealed that saturation was reached at 2,000 molecules/chloroplast. Even with less than saturating levels of complexes, a sufficient number of translocation sites could be occupied with avidin-precur sor complexes so that the import rate of freshly added radiolabeled prSS was reduced by 35%. From these observations we conclude that the trapped intermediates were blocking functional translocation sites. These biotinylated translocation intermediates should be useful in future efforts to purify and characterize the chloroplastic protein import machinery.

Most chloroplastic proteins are encoded in the nucleus and synthesized on cytoplasmic ribosomes as precursor proteins containing an N-terminal transit peptide (1–3). After translation, precursors are transported across the two envelope membranes into the stroma, where the transit peptide is removed by a stromal processing peptidase (4). It is now widely accepted that the transport process involves a proteinaceous translocation apparatus located in both the outer and inner envelope membranes of the chloroplast. Several approaches have been used to identify components of the chloroplast translocation machinery (5–10). These diverse approaches have identified several membrane proteins of 34, 70, 75, 86, and 110 kDa in molecular size (10–15). The role of these proteins in the import process remains to be elucidated. Due to the complexity of the translocation machinery, more components are probably required and remain to be identified.

Identification and characterization of components of the mitochondrial import apparatus has been aided greatly by the availability of translocation intermediates stuck in the transport machinery. This strategy has been used rather successfully by several different laboratories. For instance, a translocation intermediate was generated by Eilers and Schatz (16) using a chimeric precursor protein consisting of a mitochondrial targeting signal fused to dihydrofolate reductase (DHFR). This fusion protein can be imported into mitochondria. However, on addition of methotrexate, a folate analogue that binds with high affinity to DHFR, the fusion protein becomes stably folded and trapped in the import apparatus (16, 17). This blocked intermediate could then be cross-linked to various components of the mitochondrial import apparatus (18). In addition to identifying components of the mitochondrial import apparatus, translocation intermediates have also provided a means to investigate the energetics of protein import into mitochondria and have been useful in identifying stromal factors involved in unidirectional import into mitochondria (19).

Generating translocation intermediates using intact chloroplasts, however, has been more difficult. Limiting the levels of ATP available in an import reaction allows precursors to associate with chloroplasts but not to be fully translocated (20–22). This interaction was originally called binding, but more recently it has been recognized that it is not a reversible association, and the precursors more probably represent early translocation intermediates (8, 22). Although the topology of this early translocation intermediate is not clearly resolved (10, 40), efforts to trap precursors at a later stage of import have been less successful. For instance, the import of the precursor for 5-enolpyruvyl-shikimate-3-phosphate synthase was reduced but not arrested by the addition of its competitive inhibitor, glyphosate (23). In an approach similar to that used successfully with mitochondria, methotrexate did not block the import into chloroplasts of a chimeric fusion protein involving DHFR (24, 25). Even the introduction of a stop transfer domain from endoplasmic reticulum proteins into precursor proteins did not prevent import into chloroplasts (26). More recently, Wu et al. (15), using the chimeric precursor Oee1-DHFR, were able to generate translocation intermediates by preincubating the fusion protein with antibodies against DHFR. Schnell and Blobel (9) also generated translocation intermediates using import conditions. They devised a unique hybrid precursor consisting of...

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1 The abbreviations used are: DHFR, dihydrofolate reductase; prSS, precursor to the small subunit of ribulose bisphosphate carboxylase; BMCC, 1-biotinamido-4-[1'-4']-(maleimidomethyl)-cyclohexane-carboxamido]butane; PAGE, polyacrylamide gel electrophoresis.
prSS fused to the IgG binding domain of staphylococcal protein A. This hybrid yielded two types of translocation intermediate, depending on the conditions. Under low ATP levels the precursor formed the same type of early translocation intermediate described above. With adequate levels of ATP, the chimeric precursor imported slowly, allowing the identification of some intermediates that spanned both the outer and inner envelope membranes. The difficulty in generating translocation intermediates from chloroplast may suggest that its import machinery has different import characteristics than other transport systems.

The availability of discrete translocation intermediates blocked at later stages in import may help with the identification and characterization of additional translocation components from the inner envelope membrane and with investigating the role, if any, of molecular chaperones and other stromal factors. This article describes a strategy whereby the import of a biotinylated chloroplastic precursor is blocked when avidin is present. Available cysteine residues on prSS were modified with the biotinylation reagent biotin-BMCC (see Fig. 1). When the chemically biotinylated precursor prSS-BMCC is incubated in the presence of avidin, a complex forms. This avidin-precursor complex interacted strongly with chloroplasts but was not able to be imported when incubated with high levels of ATP. The avidin-precursor complex fractionated with the membrane fraction. This complex occupied a sufficient number of import sites to alter the import rate of freshly added precursor. The potential usefulness of these translocon intermediates for isolating components of the translocation apparatus is discussed.

EXPERIMENTAL PROCEDURES

Materials—Pea seeds (Pisum sativum var. little marlow) were obtained from Olds Seed Co. (Madison, WI). [35S]EXPRE[35S]S (cysteine/methionine) and [35S]methionine translation grade were obtained from DuPont NEN. Percoll silica gel and Mg-ATP were purchased from Sigma. Isopropyl-1-thio-β-galactosidase was obtained from Life Technologies, Inc. Biotin-BMCC, purified avidin, and immobilized avidin resin were obtained from Pierce. Plasmid pet11D-prSS (27) was provided by Dr. R. Klein (University of Kentucky, Lexington, KY).

Isolation of Chloroplasts—Intact chloroplasts were isolated from 8–12-day-old pea seedlings by homogenization and differential centrifugation followed by sedimentation through a Percoll gradient as described previously (28). Chloroplasts were washed twice in 50 mM HEPES/KOH (pH 7.7), 0.33 M sorbitol (import buffer) and finally resuspended to a concentration of 1 mg of chlorophyll/ml of import buffer.

Overexpression and Purification of prSS—The pet11D-prSS was introduced into Escherichia coli BL21(DE3) (29, 30). For [35S] labeling, prSS was prepared as described by Schnell and Blobel (9). prSS was sequestered into inclusion bodies on induction with isopropyl-1-thio-β-galactosidase. The inclusion bodies were isolated from E. coli essentially by the method of Lin and Cheng (31). Purified [35S]-prSS was stored at −80 °C at a concentration of approximately 1 mg/ml, with a specific activity of 1 × 106 dpm/μg protein.

In Vitro Transcription and Translation—An SP6 vector containing the full-length precursor of a small subunit of rubisco from pea was used for in vitro transcription and translation (32). In vitro transcription was performed using SP6 RNA polymerase to generate mRNA (26), whereas translation in the presence of [35S]methionine was performed using a wheat germ system, incubating for 90 min at 25 °C as described by Bruce et al. (25).

Biotinylation of prSS—Approximately 100 μg of E. coli-expressed [35S]-prSS in 6.0 mM guanidine-HCl, 50 mM Tris-HCl (pH 6.8), 10 mM dithiothreitol was applied to a Sephadex G-25 (Pharmacia Biotech Inc.) column equilibrated in 6.0 mM guanidine-HCl, 50 mM Tris-HCl (pH 6.8) to remove dithiothreitol. The eluant was collected and added to a biotinylation reaction containing Biotin-BMCC (Pierce) to a final concentration of 3 mM and 50 mM Tris-HCl (pH 6.8) buffer to a final volume of 100 μl. The reaction was incubated for 1–3 h at room temperature in the dark. The biotinylation reaction was quenched by the addition of dithiothreitol to 5 μmol and incubated for an additional 20 min. The reaction was diluted 10-fold with 6.0 mM guanidine-HCl, 50 mM Tris-HCl (pH 6.8) and immediately applied to a Sephadex G-25 column to remove excess biotin-BMCC. The column was centrifuged at 200 × g for 5 min. The eluant was collected and concentrated by trichloroacetic acid precipitation. The insoluble material was collected by centrifugation at 10,000 × g for 10 min. The pellet was washed two times with cold acetone, finally sonicated in 100 μl of buffer containing 6.0 mM guanidine-HCl, 50 mM Tris-HCl (pH 6.8), 10 mM dithiothreitol, and stored at −80 °C. The concentration of the biotinylated precursor protein was approximately 1 mg/ml.

Import Assay—The import of guanidine-HCl-denatured [35S]-prSS or [35S]-prSS-BMCC into isolated chloroplasts was performed as described by Perry and Keegstra (8). After import, intact chloroplasts were reisolated by centrifugation through 40% Percoll. The chloroplasts were lysed and fractionated (28) to yield a crude membrane and soluble fraction. Both membrane and soluble fractions were analyzed by SDS-PAGE and fluorography.

Import with wheat germ-translation [35S]-prSS was performed as described by Tran et al. (14). The imported proteins were analyzed by SDS-PAGE and fluorography.

Generation of Avidin-Biotinylated Precursor Complexes—Two strategies were used to generate avidin-biotinylated precursor complexes. In the first, avidin-biotinylated precursor complexes were formed prior to incubation with chloroplasts. To accomplish this, purified [35S]-prSS-BMCC expressed by E. coli was incubated in the presence of avidin in import buffer (final volume, 10–40 μl) for 5, 15, or 30 min at 25 °C in the dark. The molar ratio of biotinylated precursor:avidin in the preincubation reaction was 1:2. This ratio should favor formation of complexes with a single biotinylated precursor bound to a single avidin protein. To investigate the import capacity of precursor-avidin complexes, we added aliquots of the reaction directly to an import assay and incubated for 30 min at 25 °C. Chloroplasts were repurified through 40% (v/v) Percoll and analyzed by SDS-PAGE and fluorography.

The second strategy was first to allow biotinylated prSS to engage the translocation apparatus before avidin was added. To accomplish this, we bound [35S]-prSS-BMCC to chloroplasts at 4 °C, in the dark, for 20 min in the presence of 75 μM ATP. Intact chloroplasts were repurified through a 40% (v/v) Percoll cushion, washed twice with import buffer, and resuspended in import buffer containing avidin (at a ratio of 2 mol of avidin:1 mol of biotinylated precursor). Complex formation continued at 4 °C, in the dark, with continuous rocking for 30 min. Import for 30 min was initiated by adjusting the ATP concentration to 4 mM and the temperature to 25 °C. Chloroplasts were repurified through 40% (v/v) Percoll and analyzed by SDS-PAGE and fluorography.

Titration of Import Sites with Avidin-Precursor Complexes—Increas-

ing amounts of E. coli-expressed precursor (15–245 nmol) were incubated with chloroplasts at 75 mM ATP in the dark at 4 °C for 10 min. Chloroplasts were repurified through a 40% Percoll cushion, washed with import buffer, and then incubated with import buffer containing avidin. Complex formation continued for an additional 30 min, in the dark, on ice. Import was then initiated by adjusting ATP levels to 4 mM and allowed to continue for 20 min at room temperature in room light. The entire reaction was overlaid onto a 40% Percoll cushion followed by centrifugation. Intact chloroplasts recovered from the pellet were fractionated into a crude membrane and supernatant fraction. The membrane fraction containing the blocked avidin complexes were analyzed by SDS-PAGE and fluorography. Gels were further quantitated directly by PhosphorImager (Molecular Dynamics).

Competition Experiment using Avidin-Precursor Complexes—Chloroplasts containing avidin-biotinylated precursor complexes trapped in the import apparatus were prepared as described in the previous section and repurified through a 40% Percoll cushion. Chloroplasts were resuspended in 400 μl of import buffer containing 4 mM Mg-ATP and allowed to incubate at room temperature for 15 min. Wheat germ-translated prSS (~500,000 dpm/100-μl reaction) was added, and import proceeded for 0, 5, 2, 4, and 20 min. Import reactions were immediately terminated at the times indicated by diluting 10-fold with cold import buffer and immediately centrifuging through a 40% Percoll cushion. Pellets were resuspended in sample buffer and analyzed by SDS-PAGE and fluorography. Gels were quantitated directly by PhosphorImager (Molecular Dynamics).

RESULTS

Biotinylation of prSS and Examination of its Import into Pea Chloroplasts—The cysteine residues of chemically purified prSS were modified by reaction with biotin-BMCC (see Fig. 1). Biotinylation of prSS was confirmed by SDS-PAGE and detection of the biotinylated proteins with avidin conjugated to alkaline phosphatase (Fig. 2A,
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FIG. 1. Constructs used in this investigation. prSS was used as a control protein that was not biotinylated. Biotinylated precursor, prSS-BMCC, represents a small subunit of rubisco that has been chemically modified at available cysteine residues by the biotinylation reagent BMCC. ©, location of the cysteines available for biotinylation.

![Constructs diagram](image)

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prSS
- Transil peptide
- Mature SS

prSS-BMCC
- Transil peptide
- Mature SS

© = Biotin-BMCC

FIG. 2. Biotinylation of prSS using BMCC. Purified E. coli-expressed 35S-labeled prSS was biotinylated using the reagent BMCC. A, biotinylation of prSS was confirmed by probing a blot with avidin conjugated to alkaline phosphatase. Lane 1, prSS; lane 2, biotinylated prSS, designated prSS-BMCC. B, fluorogram generated from the blot in A demonstrates that after biotinylation of prSS a shift in mobility can be observed between prSS and prSS-BMCC. MW, molecular weight markers.

![Biotinylation diagram](image)

Fig. 2 shows the biotinylation of prSS using BMCC. Purified E. coli-expressed 35S-labeled prSS was biotinylated using the reagent BMCC. A, biotinylation of prSS was confirmed by probing a blot with avidin conjugated to alkaline phosphatase. Lane 1, prSS; lane 2, biotinylated prSS, designated prSS-BMCC. B, fluorogram generated from the blot in A demonstrates that after biotinylation of prSS a shift in mobility can be observed between prSS and prSS-BMCC. MW, molecular weight markers.

![ Autorad diagram](image)

![Blot diagram](image)

FIG. 3. Import of prSS and prSS-BMCC into isolated pea chloroplasts. Either prSS or prSS-BMCC was incubated in the presence of pea chloroplasts, and import reactions were stopped after 30 min. Intact chloroplasts were repurified with pelleting through a 40% Percoll cushion. The recovered chloroplasts were treated with thermolysin for 30 min on ice, and intact chloroplasts were repurified through a Percoll cushion, lysed, and fractionated into membrane (P) and soluble (S) fractions. Samples were analyzed by 12.5% SDS-PAGE and fluorography. O.P., 20% of overexpressed protein added to an import reaction; MW, molecular weight markers.

![Temperature chart](image)

![Thermolysin time chart](image)

![MW chart](image)

![MW chart](image)

![MW chart](image)

Biotinylated prSS can be imported into intact pea chloroplasts (Fig. 3). Protease protection assays using thermolysin confirmed that both mature-sized SS and SS-BMCC were no longer sensitive to digestion and therefore had entered chloroplasts (Fig. 3, lanes 3 and 6, respectively). Both SS and SS-BMCC were present in the soluble fraction after import (Fig. 3, lanes 3 and 6, respectively). Moreover, the mobility of imported SS-BMCC was similar to that of SS. However, the modified SS still contained biotin, because on analysis by SDS-PAGE, fol-

However, biotinylated prSS was not processed by the stromal peptidase when avidin was present. From this result, we conclude that complexes of avidin and biotinylated precursors do not extend through the import apparatus sufficiently far to gain access to the stroma processing protease. Indeed, it is technically possible, although unlikely, that the complexes of avidin and biotinylated precursors were not specifically associating with the import apparatus but, rather, were simply aggregating at the surface of chloroplasts. To address this concern, biotinylated precursors were bound to chloroplasts before the addition of avidin.

Fig. 5 shows results from such an experimental approach.

In the experiment shown in Fig. 4, avidin was first incubated with biotinylated precursors for 5, 15, or 30 min before the complexes were added to chloroplasts. The data in Fig. 4 show that these complexes bound to chloroplasts; however, they were not imported (Fig. 4, compare lane 8 with lanes 9, 11, and 13). In control experiments, biotinylated prSS was imported in the absence of avidin, (Fig. 4, lane 8), and the import of unmodified prSS was not blocked by avidin (Fig. 4, compare lanes 3 and 5). Complexes of avidin-biotinylated prSS fractionated with membranes (Fig. 4, lanes 9, 11, and 13). In control experiments, the products derived from biotinylated prSS imported in the absence of avidin (Fig. 4, lane 8) and SS imported with or without avidin (Fig. 4, lanes 3 and 5) were present in the soluble fraction. The simplest explanation is that biotinylated prSS is trapped in the translocation apparatus when avidin is present.

However, biotinylated prSS was not processed by the stromal peptidase when avidin was present. From this result, we conclude that complexes of avidin and biotinylated precursors do not extend through the import apparatus sufficiently far to gain access to the stroma processing protease. Indeed, it is technically possible, although unlikely, that the complexes of avidin and biotinylated precursors were not specifically associating with the import apparatus but, rather, were simply aggregating at the surface of chloroplasts. To address this concern, biotinylated precursors were bound to chloroplasts before the addition of avidin.

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Fig. 4. Import competence of avidin-prSS-BMCC complexes. Biotinylated precursors were incubated with avidin (1:2 molar ratio). Aliquots from this preincubation reaction were removed at 5, 15, and 30 min and added directly to chloroplasts in import buffer containing 4 mM Mg-ATP and incubated for 30 min at 25 °C. Control reactions with unmodified prSS were performed in a similar manner. Intact chloroplasts were repurified through a 40% Percoll cushion, lysed, and fractionated into membrane (P) and soluble (S) fractions. These fractions were analyzed by 12.5% SDS-PAGE and fluorography. OP, 20% of overexpressed protein added to an import reaction; MW, molecular weight markers; − and +, absence and presence of avidin, respectively.

Fig. 5. Avidin can prevent the import of prSS-BMCC prebound to chloroplasts. Either prSS or prSS-BMCC was incubated with chloroplasts at 4 °C, in the dark, in the presence of 75 μM Mg-ATP. Chloroplasts were repurified through a 40% Percoll cushion. The chloroplastic pellet was washed twice with import buffer and finally resuspended in import buffer containing avidin. Complex formation was allowed to occur for 30 min at 4 °C, in the dark, with continuous rocking. Import was initiated by adjusting the Mg-ATP levels to 4 mM and was continued for an additional 30 min at room temperature. Chloroplasts were repurified through a 40% Percoll cushion, lysed, and fractionated into membrane (P) and soluble (S) fractions as described under “Experimental Procedures.” These fractions were analyzed by 12.5% SDS-PAGE and fluorography. − and +, absence and presence of avidin, respectively; OP, 20% of overexpressed protein added to an import reaction; MW, molecular weight markers.

Import of native prSS from the prebound state was not affected by the presence of avidin (Fig. 5, lane 3); however, in the presence of avidin, import of biotinylated prSS import was blocked (Fig. 5, lane 7). Complexes of avidin-biotinylated prSS assembled from the prebound state fractionated with membranes. In the absence of avidin, prebound biotinylated prSS was imported into chloroplasts and fractionated to the soluble portion (Fig. 5, lane 6). From these results we conclude that prebinding of biotinylated precursor to chloroplasts does not prevent avidin from interacting with available biotinylated cysteines within the precursor. However, it is uncertain which biotinylated cysteine is interacting with avidin.

Titration of Import Sites using Avidin-Biotinylated Precursor Complexes—Import intermediates can serve as valuable tools for determining the number of import sites present on a chloroplast. Because binding of biotinylated precursors is specific, as demonstrated by their nearly complete import on addition of ATP (Fig. 5, lane 6), the complexes formed with these precursors should be useful to titrate chloroplastic import sites. Import sites became saturated with increasing amounts of avidin-biotinylated precursor complexes (Fig. 6A). The quantities of avidin-biotinylated prSS complexes that associated with the membrane fraction were measured, and the resulting data were graphed (Fig. 6B). The number of complexes reached saturation at approximately 2,000 molecules/chloroplast (Fig. 6B). In a control experiment, biotinylated prSS at a high concentration (245 nM) in the absence of avidin was imported into chloroplasts and was present in the soluble fraction (data not shown). This control confirms that association of biotinylated precursors with membranes was a result of avidin complex formation and not due to aggregation.

Avidin-Precursor Complexes Inhibit the Import of Wheat Germ-translated prSS—The results presented above suggest that under import conditions avidin-precursor complexes can initiate translocation but become blocked in import sites because of the presence of a bulky avidin protein. If partially translocated avidin complexes remain stably associated with the import machinery, the limited number of import sites of a chloroplast will eventually be inactivated, leading to a reduction in the capacity of chloroplasts to import other precursor proteins. To examine this possibility, chloroplasts containing trapped avidin-biotinylated precursor complexes were incubated with wheat germ-translated prSS, and its rate of import was monitored. Chloroplasts were incubated with 79 nM biotinylated prSS, and avidin complexes formed as described in Fig. 6. When radioactively labeled prSS was examined, its import was inhibited (Fig. 7A, compare rows 4 and 1). Fig. 7B quantitates data in Fig. 7A and shows that the rate of import of prSS is reduced by approximately 35% when import sites are blocked with avidin-biotinylated precursor complexes (Fig. 7B, compare lines 1 and 4). This inhibition was dependent on the formation...
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The successful generation of translocation intermediates provides an effective tool in dissecting the process by which proteins traverse the chloroplastic envelope membranes. Translocation intermediates can be used to identify, characterize, and purify components of the import apparatus in addition to delineating individual steps in the import process. In the present investigation, we have used biotinylated precursors to generate translocation intermediates that block protein import into chloroplasts. We have demonstrated that avidin-precursor complexes could be formed by adding avidin to biotinylated precursors prebound to the import machinery. The bulky avidin complex was unable to be translocated through the transport machinery, resulting in obstructed import sites. The effectiveness of this obstruction was shown when freshly translated prSS was added to chloroplasts containing obstructed import sites. Our titration experiment showed that a 79 nM concentration of biotinylated precursor was sufficient to occupy approximately 800 translocation sites per chloroplast once avidin was added (Fig. 6A). This concentration should result in occupation of avidin-precursor complexes, because the rate of import of prSS was not affected when avidin alone (Fig. 7, A, row 2, and B, line 2) or biotinylated prSS (Fig. 7, A, row 3, and B, line 3) were incubated independently. From these results, we conclude that avidin-biotinylated precursor complexes remain specifically associated with import sites that are also used by prSS. Blockade of the import apparatus by these complexes prevents the import of prSS into chloroplasts.

**DISCUSSION**

Others have used different strategies to estimate the number of binding sites located on a chloroplast. Using wheat germ-translated prSS, Friedman and Keegstra (33) demonstrated that saturation of binding occurred at 1,500–3,000 precursors/chloroplast. Schnell and Blobel (9), when using the urea-denatured fusion protein prSS/protein A, calculated that saturation of binding was obtained at 2,000 precursors/chloroplast. We have attempted to use halted complexes to calculate the number of translocation sites per chloroplast (Fig. 6B). After quantitating membranes with blocked avidin complexes, we calculated that saturation occurred at approximately 2,000 precursors/chloroplast (Fig. 6B). This is in agreement with the results of Friedman and Keegstra (33) and Schnell and Blobel (9). However, the number of precursors associated with a chloroplast, as reported by Friedman and Keegstra (33) and Schnell and Blobel (9), represents a combination of binding and early transport intermediates. In binding experiments precursors interact with both surface receptor(s) and components of the import apparatus. Halted avidin-precursor complexes, however, represent precursors that have exclusively engaged the import apparatus at a later stage in import. The level of saturation calculated from our titration experiments therefore represents the number of translocation sites present per chloroplast. Studies with translocation intermediates using mitochondria have estimated that the number of import sites is $10^8$–$10^9$/mitochondrion (17, 34). Another study using an avidin fusion protein to generate translocation intermediates calculated approximately 600 import sites/mitochondrion (35).

The translocation intermediates generated cannot be chased into the stroma even under import conditions. This is in contrast to the translocation intermediate generated by Schnell and Blobel (9) using a chimeric precursor protein containing portions of prSS fused to protein A. In their studies a translocation intermediate was observed early during the time course of import, but at later times the fusion protein was chased into the stroma. Thus the approach reported here provides a means for generating permanently arrested translocation intermediates, whereas the approach of Schnell and Blobel (9) produced a slowly imported chimeric precursor that allowed a transitory translocation intermediate to be identified.

Our experiments also revealed that biotinylated precursors can serve as a valuable tool for examining and isolating the chloroplastic import machinery. Modification of precursors with biotin does not dramatically affect their capacity for spe-
cific binding to chloroplast. Import likewise does not appear to be greatly impaired. However, processing of newly imported biotinylated prSS appears to be aberrant, since the mobilities of SS-BMCC and SS are similar (Fig. 3, compare lanes 3 and 6). Of the four cysteines within prSS that can be modified with biotin-BMCC, one is located near the cleavage site for the transit peptide (see Fig. 1). This particular modification appears to result in aberrant processing of prSS-BMCC. This result is not surprising; numerous studies on chloroplastic transit peptides in which various amino acid residues have been mutated near the cleavage site have yielded similar reports of aberrant processing (36–38). Robinson and Ellis (39) demonstrated that when cysteines on prSS are modified with iodoacetic acid, import is not affected, but processing is aberrant. However, we cannot exclude other possible explanations for the similar mobility of SS and SS-BMCC. Regardless of this uncertainty, the utility of biotinylated precursors is not diminished.

Biotinylated precursors should be useful in identifying and isolating components of the chloroplastic import machinery. One strategy would entail binding biotinylated precursors to the translocation apparatus, followed by purification of translocation complexes by affinity chromatography using immobilized avidin. This and other possibilities for investigating the import process using biotinylated precursors are underway.

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