Three Sets of Translocation Intermediates Are Formed during the Early Stage of Protein Import into Chloroplasts*

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During the early stage of protein import into chloroplasts, precursor proteins synthesized in the cytosol irreversibly bind to chloroplasts to form the early translocation intermediate under stringent energy conditions. Many efforts have been made to identify the components involved in protein import by analyzing the early intermediate. However, the state of the precursor within the intermediate has not been well investigated so far. In this study, an attempt was made to evaluate the extent of translocation of the precursor by determining the state of the precursor in the early intermediate under various conditions and analyzing the fragments generated by limited proteolysis of the precursors docked to chloroplasts. Our results indicate that three different sets of early intermediate are formed based on temperature and the hydrolysis of GTP/ATP. These have been identified based on the size of proteolytic fragments of the precursor as “energy-dependent association,” “insertion,” and “penetration” states. These findings suggest two individual ATP-hydrolyzing steps during the early stage of protein import, one of which is temperature-sensitive. Our results also demonstrate that translocation through the outer envelope membrane is mainly dependent on internal ATP.

Most chloroplastic proteins are encoded in the nuclear genome. The majority of these proteins are synthesized as precursors with chloroplastic targeting signals, the transit peptides, at their amino termini and are considered to translocate through the general import pathway. The general import pathway is composed of translocons at the outer and the inner envelope membranes, the Toc and the Tic complexes, respectively (for review, see Ref. 1). Many components of the Toc and Tic complexes have been reported and are being characterized biochemically and genetically by various research groups.

In vitro studies indicate that the process of protein import into chloroplasts consists of two energy-dependent stages, “docking” and “translocation.” In “docking,” the precursor proteins irreversibly bind to chloroplasts to form the early translocation intermediates. A low level of ATP (less than 0.1 mM) is required for this step (2). A low level of GTP also supports this step and enhances ATP-dependent docking, but GTP is unable to substitute for ATP (2–4). The early intermediate is also observed at low temperatures even at higher ATP concentrations (5, 6). In “translocation,” which requires a higher level of ATP (more than 1 mM) at higher temperatures, precursors translocate across both the envelope membranes, and their transit peptides are cleaved by the stromal processing peptidase (7, 8). The requirement of GTP for docking is accounted for by the two GT-Pases in the Toc complex, Toc159 and Toc34, regarded as the receptors for incoming precursors (9). On the other hand, the requirement of a low level of ATP for docking is far from understood. It is possible that the intermembrane space molecular chaperone, HSP70-IAP (10), may be contributing to the ATP requirement cooperating with Toc12, which contains the J-domain (11).

Many of the Toc and Tic components have been isolated and identified from the early intermediates by incubating precursors with isolated chloroplasts at different temperatures in the presence of GTP and/or ATP (10, 12–16). Previous reports suggest that precursors reach deeper in the early intermediate stage at 25 °C rather than at 4 °C in the presence of 0.1 mM ATP (17); however, direct comparisons of the early intermediates formed under different conditions have never been attempted. Hence, the following questions have remained unsolved. How far does the precursor reach in the intermediate formed under different conditions? Are similar intermediates formed in the presence of GTP and ATP? Does temperature affect the intermediate formation? In this report, the authors have attempted to evaluate the early intermediates by assessing the location of precursors in the intermediates formed under different conditions.

To conduct the docking reaction under stringent conditions, purified recombinant precursors, free from any cytosolic factors, were used. These precursors were applied to the in vitro protein import assay system to estimate the extent of translocation in the early intermediate that was formed under various conditions, and the outer surface of the chloroplast was subjected to limited proteolysis. Analysis of the undigested fragments of the precursor protected by the outer envelope membrane showed the presence of different sets of intermediates based on their requirement for GTP/ATP and temperature. Each set consisted of three different types of intermediates with precursors in different stages of translocation, indicated by the sizes of the proteolytic fragments: “energy-dependent association,” “insertion,” and “penetration” states. The finding that transition from one to the forward step required ATP hydroly-
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sis indicates that two individual ATP hydrolysis steps are present during the early stage of protein import.

EXPERIMENTAL PROCEDURES

Growth of Plants and Isolation of Chloroplasts—Pea (Pisum sativum) plants were grown for 8–10 days in a growth chamber under a cycle of 14 h of light at 24 °C and 10 h of dark at 20 °C. Intact chloroplasts were isolated as described earlier (18). After isolation, chloroplasts were suspended in import buffer (I-buffer\(^3\)): 50 mM HEPES-KOH, pH 7.0, 300 mM NaCl, 20 mM imidazole. The precursor proteins were further purified from inclusion bodies on Ni\(^2+\)-NTA-agarose (Qiagen, Hilden, Germany). Solubilized inclusion bodies and resins pre-equilibrated with denaturing/wash buffer were mixed in a microtube and incubated for 20 min at room temperature with frequent mixing. After recovery of resin by centrifugation and the proteolytic fragments were quantified from the digests, images were analyzed by UN-SCAN-IT version 5.1 (Silk Scientific, Orem, UT).

Preparation of Recombinant Precursor Protein—Expression plasmid harboring mutant types of precursors (Fig. 1A) was prepared by site-directed mutagenesis from pET8c-pS (19). E. coli BL21(DE3) carrying the plasmid was incubated at 37 °C until midlogarithmic phase when 0.4 mM isopropyl 1-thio-β-D-galactopyranoside was added to overexpress the precursor and then incubated for 4 h at 37 °C. Recombinant protein was recovered in inclusion bodies. After several washes with \(\text{H}_2\text{O}\), inclusion bodies were solubilized in denaturing/wash buffer (8 mM urea, 50 mM HEPES-KOH, pH 7.0, 300 mM NaCl, 20 mM imidazole). The precursor proteins were further purified from inclusion bodies on Ni\(^2+\)-NTA-agarose (Qiagen, Hilden, Germany). Solubilized inclusion bodies and resins pre-equilibrated with denaturing/wash buffer were mixed in a microtube and incubated for 20 min at room temperature with frequent mixing. After recovery of resin by centrifugation (700 \(\times\) g, 5 min at room temperature), resins were washed with denaturing/wash buffer twice. Precursor protein bound to resins was eluted with elution buffer (7.2 mM urea, 45 mM HEPES-KOH, pH 7.0, 270 mM NaCl, 200 mM imidazole). Precursor protein was then precipitated with 10% trichloroacetic acid to remove imidazole, followed by washes with acetone and ethanol. After drying the precipitate, precursor protein was dissolved in solubilization buffer (S-buffer: 8 mM urea-25 mM HEPES-KOH, pH 7.5–50 mM KCl-2 mM MgCl\(_2\)). Protein was determined by the method of Lowry (20), and the precursor was suspended in S-buffer at a concentration of 0.1 mM.

Biotinylation of the Precursor—To modify precursor proteins with biotin, 2.5 mM biotin-maleimide (Sigma) was added to 100 \(\mu\)M precursor in S-buffer, and the mixture was incubated for 1–4 h on ice in the dark. The reaction was quenched by the addition of 5 mM dithiothreitol, and the mixture was further incubated for 15 min on ice. Excess biotin-maleimide was removed by trichloroacetic acid precipitation, and the final pellet was solubilized in S-buffer and stored in the freezer until use.

The Docking and the Import Reaction—Isolated chloroplasts were incubated on ice in the dark for 1 h and then diluted twice with 5 \(\mu\)M nigericin in I-buffer and incubated for 10 min at 25 °C in the dark. Chloroplasts were then diluted 5-fold with 1-buffer containing 1 mg/ml bovine serum albumin, 25 mM dithiothreitol, 5 mM MgCl\(_2\), 0.1 mM GTP, and/or 0.1 mM ATP incubated at 4 or 25 °C for 5 min in the dark. The docking reaction was initiated by the addition of 200 nm precursor in S-buffer and incubated at either 4 or 25 °C for 5 min in the dark. Chloroplasts were recovered by centrifuging (1,500 \(\times\) g) the reaction mixture on a 40% cushion of Percoll in I-buffer at 4 °C for 5 min and washed once with I-buffer.

Protease Treatment—After recovery of chloroplasts, they were treated with thermolysin or trypsin. Recovered chloroplasts were suspended (1 mg of chlorophyll/ml) in I-buffer with 0.8 mg of thermolysin/mg of chlorophyll in 4 mM CaCl\(_2\) or 0.1 mg of trypsin/mg of chlorophyll in 1 mM CaCl\(_2\) and incubated for 30 min on ice in the dark, unless stated otherwise. The mixture was diluted 4-fold in I-buffer in the presence of protease inhibitors to quench proteolysis and incubated for a further 10 min on ice. The following protease inhibitors were used for quenching: 12 mM EDTA for the thermolysin treatment; 1 mM phenylmethylsulfonyl fluoride, 0.05 mg/ml N\(^{\text{carboxyamidomethyl}}\)-p-tosyl-L-lysine chloromethyl ketone, 2 \(\mu\)g/ml aprotinin and 0.1 mg/ml soybean trypsin inhibitor for the trypsin treatment. After quenching, intact chloroplasts were resolated through a 40% Percoll cushion containing the same protease inhibitors and washed with I-buffer containing inhibitors. The resulting pellet was suspended in SDS-PAGE sample buffer of Laemmli’s system (21) containing 1 mM EDTA for thermolysin-treated samples or 1 mM PMSF for trypsin-treated samples and immediately boiled for 10 min.

Electrophoresis, Immunoblotting, and Quantification—Proteolytic fragments were analyzed by SDS-PAGE in the Tris-Tricine-buffered system (22) on 16.5% polyacrylamide gel, and other samples were analyzed by Laemmli’s SDS-PAGE (21) on 7.5–15% polyacrylamide gradient gels. Blotting was performed as described earlier (14). After blotting, polypeptides on the blots were decorated with the antibodies against the respective polypeptides, followed by the alkaline phosphatase (AP)-conjugated secondary antibodies. Biotinylated polypeptides on the blots were decorated with AP-conjugated streptavidin. Color development by AP was carried out by soaking the blots in 33 ng/ml nitro blue tetrazolium and 8.3 ng/ml 5-bromo-4-chloro-3-indolyl phosphate in AP-buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl\(_2\), pH 9.5) until optimal color development occurred. The blots were washed in \(\text{H}_2\text{O}\) and dried for storage. Images of the blots were scanned, and then the docked precursor and the proteolytic fragments were quantified from the digitized images by UN-SCAN-IT version 5.1 (Silk Scientific, Orem, UT).

Sources of Antibodies—Except for the monoclonal antibody against HA tag produced in mouse clone HA-7 (Sigma), all antibodies used in this study were rabbit polyclonal and were a gift from Dr. Ken Keegstra. For the detection of proteolytic fragments, AP-conjugated streptavidin was used (Invitrogen).

RESULTS

Experimental Design—A precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase (prSS) in modified form, overexpressed in E. coli cells, was used as the import substrate (Fig. 1A). Recombinant precursor was recovered in inclu-
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A. The wild-type prSS has a 57-amino acid-long transit peptide (indicated as TP) and 3 cysteine residues (indicated as X) at positions −1, −41, and +112. The plasmid carrying the wild-type prSS was manipulated to carry a single cysteine at position −52 fused with a double-HA (HAHA) tag and hexahistidinyl (H) tag (designated as prSSC1(−52)HAHAH). This expression plasmid was overexpressed in E. coli cells. Overexpressed prSSC1(−52)HAHAH, recovered in the inclusion bodies, was solubilized with urea-containing buffer and purified as described under “Experimental Procedures” for further experiments. B, formation of the proteolytic conditions. Chloroplasts, incubated with 5 μM nigericin at 25 °C for 10 min in the dark, were incubated with prSSC1(−52)HAHAH, biotinylated by biotin-maleimide, in the presence of 0.1 mM ATP and 0.1 mM GTP at 4 °C for 5 min. To avoid the light-mediated precursor docking, chloroplasts were incubated with nigericin to inhibit ATP synthesis generated by F0F1-ATPase embedded in the thylakoid membrane by uncoupling electron transport through the thylakoid membrane (23). All further experiments were performed with chloroplasts treated with nigericin. Approximately 5–8% of the precursor used in the docking reaction was bound to chloroplasts (Fig. 1B, lane 2 in αHA and lane 12 in St). Biotinylation did not affect the interaction between the precursor and chloroplasts4 or protein import4 (24).

Proteolytic fragments were analyzed by Tris-Tricine-buffered PAGE, since this electrophoresis system efficiently separates small polypeptides (22). However, in this PAGE system, the biotinylated precursor was hindered by one of the two endogenous streptavidin-reactive proteins in chloroplasts (Fig.

B. Limited proteolysis of early translocation intermediate. A, diagrams of the precursor used in this study. The wild-type prSS has a 57-amino acid-long transit peptide (indicated as TP) and 3 cysteine residues (indicated as X) at positions −1, −41, and +112. The plasmid carrying the wild-type prSS was manipulated to carry a single cysteine at position −52 fused with a double-HA (HAHA) tag and hexahistidinyl (H) tag (designated as prSSC1(−52)HAHAH). This expression plasmid was overexpressed in E. coli cells. Overexpressed prSSC1(−52)HAHAH, recovered in the inclusion bodies, was solubilized with urea-containing buffer and purified as described under “Experimental Procedures” for further experiments. B, determination of the proteolytic conditions. Chloroplasts, incubated with 5 μM nigericin at 25 °C for 10 min in the dark, were incubated with prSSC1(−52)HAHAH, biotinylated by biotin-maleimide, in the presence of 0.1 mM ATP and 0.1 mM GTP for 5 min in the dark to form the early translocation intermediate at 4 °C (lanes 2-8, 12, and 13). After recovery of intact chloroplasts through a 40% Percoll, chloroplasts were treated with 0.8 mg (lanes 3-5 and 10) or 1.6 mg (lanes 6-8) of thermolysin/mg chlorophyll equivalent of chloroplasts at 4 °C for the times indicated (lanes 3-8 and 13). Chloroplastic proteins, the docked precursor, and the proteolytic fragments generated from the precursor were analyzed by SDS-PAGE either in the Tris-glycine buffer system (21) or in the Tris-Tricine buffer system (22), followed by blotting. HA tag-containing polypeptides on the blots were decorated with the monoclonal antibody against HA (αHA), whereas Toc75 and Toc34 on the blots were decorated with polyclonal antibodies against Toc75 (αToc75), and Toc34 (αToc34), respectively. Biotinylated polypeptides were probed with streptavidin (St). Detection of these polypeptides was described under “Experimental Procedures.” 10% of precursor in the reaction mixture (pr) was loaded onto the gel (lanes 1 and 11). As controls, the precursor-docked chloroplasts without thermolysin treatment (lanes 2 and 12) and chloroplasts incubated without the precursor treated either with (lane 10) or without thermolysin (lane 9) at 4 °C for 30 min were also analyzed. Two of the streptavidin-reactive endogenous proteins in chloroplasts are indicated as the shaded diamond and the open diamond. The image taken from the same blots but from different areas was separated by dotted vertical lines. C, the specificity of proteolytic fragments generated from the early translocation intermediate. After the docking reaction was performed with nigericin-pretreated chloroplasts as described above (lanes 2-4), chloroplasts were treated with either 0.8 mg of thermolysin (lane 3) or 0.1 mg of trypsin/mg of chlorophyll equivalent of chloroplasts (lane 4) at 4 °C for 30 min. The precursor alone (lanes 5-7) was also treated with the same concentration of thermolysin (lane 6) or trypsin (lane 7) at 4 °C for 30 min. Chloroplastic proteins and the docked precursor were analyzed by SDS-PAGE in the Tris-glycine buffer system (21), whereas the proteolytic fragments generated from the precursor were analyzed by SDS-PAGE in the Tris-Tricine buffer system (22), followed by blotting as described above. 10% of the precursor applied for the docking reaction (lane 1) was loaded onto the gel, designated as pr. As controls, the precursor-docked chloroplasts without protease treatment (lane 2) and the precursor without protease treatment (lane 5) were also analyzed by SDS-PAGE. Biotinylated prSS is indicated by the filled arrowhead. The final proteolytic fragments generated by the thermolysin and the trypsin treatment are indicated by the open circles and squares, respectively.

4 H. Inoue and M. Akita, unpublished observations.
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1B, lane 2 in St). The precursor was completely separated from the endogenous protein in the Laemmli Tris-glycine-buffered PAGE system (Fig. 1B, lane 12 in St).

After chloroplasts were recovered, they were treated with 0.8 mg of thermolysin/mg of chlorophyll equivalent of chloroplasts at 4 °C. Thermolysin is known to digest the outer surface of chloroplasts (25). After 5 min of proteolysis, prSS disappeared completely (Fig. 1B, lane 3 in αHA) and smaller fragments (less than 25 kDa) started to appear (Fig. 1B, lanes 3 in St). These fragments were detected only by streptavidin (Fig. 1B, lanes 3–8 in St), and no fragment was detected by the anti-HA antibody,4 indicating that these fragments were derived from the amino-terminal part of the precursor. As the proteolytic period was increased, these fragments were degraded further, and four major fragments (open circles), with molecular masses between 7.8 and 12 kDa, were observed after 30 min of proteolysis (Fig. 1B, lanes 3–5 in St). When the concentration of thermolysin was increased 2-fold, these four fragments did not degrade further (Fig. 1B, lanes 6–8 in St). These fragments were not observed when the precursor was treated with thermolysin in the absence of chloroplasts, demonstrating that they were not representing the protease-resistant domains of the precursor (Fig. 1C, lanes 5–7 in St).

Accessibility of thermolysin was evaluated by monitoring two outer envelope membrane proteins at the same time. The outer envelope membrane protein, Toc34, a large amino-terminal domain facing the cytosol, was completely degraded after 5 min of thermolysin treatment (Fig. 1B, lane 3 in αToc34). On the other hand, the integral outer envelope membrane protein, Toc75, remained intact even after 30 min of proteolysis (Fig. 1B, lanes 5 and 8 in αToc75).

Two proteolytic fragments (open squares) were generated from the precursor docked to chloroplasts treated with 0.1 mg of trypsin/mg of chlorophyll equivalent of chloroplasts at 4 °C for 30 min (Fig. 1C, lane 4). The molecular masses of trypsin-generated proteolytic fragments were observed in the same molecular mass range as those produced by thermolysin (Fig. 1C, lane 3). With the trypsin treatment, two Toc components, Toc34 and Toc75, displayed the same behavior as with the thermolysin treatment (Fig. 1C, lanes 3 and 4). The similar sizes of proteolytic fragments, generated from the biotinylated precursor at −52, were also observed when the biotinylated precursor carrying the cysteine residue at position −33 or +4 was treated by these proteases.4 This observation, along with the size of the proteolytic fragments, indicates that the entire part of the transit peptide completely crossed the outer envelope membrane under this docking condition.

Proteolytic Fragments Were Produced by the Import-competent Precursor Associating with the Membranes—Since proteolytic fragments were observed only when the precursor was incubated with chloroplasts, we further verified the docking specificity of the precursor. The precursor docked to chloroplasts in the presence of 0.1 mM GTP and 0.1 mM ATP at 4 °C was chased with 2.5 mM ATP at 25 °C. Following the chase, the docked precursor (Fig. 2A, lane 3 in αHA) was decreased significantly, and the protease-resistant mature form of the small subunit of ribulose-1,5-bisphosphate carboxylase (mSS) was observed (Fig. 2A, lanes 4 and 7 in αHA). Since the

FIGURE 2. Proteolytic fragments are generated from the early translocation intermediate (A) and localized in the membranes (B). 4 A, the docking reaction was performed with nigericin-pretreated chloroplasts, as described in the reference to Fig. 1 (indicated as Do; lanes 3 and 6). After recovery of chloroplasts, chloroplasts were incubated at 25 °C for 20 min in the presence of 2.5 mM ATP to chase the precursor (Ch; lanes 4 and 7). After the docking reaction or the chase experiment, chloroplasts were treated either with (lanes 5–7) or without (lanes 2–4) thermolysin (0.8 mg/mg of chlorophyll) for 30 min at 4 °C. Chloroplastic proteins and the docked precursor were analyzed by SDS-PAGE in the Tris-glycine buffer system (21), whereas the proteolytic fragments generated from the precursor were analyzed by SDS-PAGE in the Tris-Tricine buffer system (22), followed by blotting, as described in the legend to Fig. 1. 10% of precursor in the reaction mixture was loaded onto the gel (pr; lane 1), B, after the docking reaction was performed with nigericin-pretreated chloroplasts as described in Fig. 1, followed by the recovery of chloroplasts, chloroplasts were treated either with thermolysin (0.8 mg/mg of chlorophyll; lanes 4 and 5) or with trypsin (0.1 mg/mg of chlorophyll; lanes 6 and 7) at 4 °C for 30 min. Then chloroplasts were lysed hypertonically and separated into the soluble (S; lanes 2, 4, and 6) and membrane (P; lanes 3, 5, and 7) fractions by ultracentrifugation (100,000 × g, 30 min at 4 °C). Chloroplastic proteins and the docked precursor were analyzed by SDS-PAGE in the Tris-glycine buffer system (21), whereas the proteolytic fragments generated from the precursor were analyzed by SDS-PAGE in the Tris-Tricine buffer system (22), followed by blotting as described above. 10% of precursor in the reaction mixture was loaded onto the gel (pr; lane 1). Positions of prSS and mSS are indicated by the filled and the open arrowhead, respectively. The proteolytic fragments generated by the thermolysin and the trypsin treatment are indicated by the open circles and squares, respectively.
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Proteolytic fragments also disappeared following the chase (Fig. 2A, lane 7 in St). From these results, it can be concluded that the precursor was bound specifically to chloroplasts and that the chloroplast-docked precursor maintains import competence.

Because proteolytic fragments were recovered with chloroplasts, they were expected to be localized in the membranes. Thus, their location was determined. After proteolysis, chloroplasts were lysed hypertonically and fractionated into soluble and insoluble fractions (Fig. 2B). The docked precursor (Fig. 2B, lane 3 in αHA) and proteolytic fragments (open circles and squares in Fig. 2B, lanes 5 and 7 in St) were observed only in the insoluble fraction, demonstrating that proteolytic fragments were recovered in the membranes. These results demonstrate that the experimental procedure in the present study was effective in analyzing the precursor in the early intermediates. Hence, the conditions for proteolysis were set at 0.8 mg of thermolysin/mg of chlorophyll equivalent of chloroplasts for 30 min at 4 °C.

Limited Proteolysis of the Early Translocation Intermediate Formed under Different Energy Conditions— Once the proteolytic conditions were set, the early translocation intermediates formed under different conditions were analyzed. Biotinylated prSSC1(−52)HAHAH was incubated with nigericin-pretreated chloroplasts in the presence of different concentrations of GTP and/or ATP either at 4 °C (A, B, E, and F) or at 25 °C (C, D, G, and H) for 5 min, followed by thermolysin (0.8 mg/mg chlorophyll) treatment at 4 °C for 30 min. The docked precursor was analyzed by SDS-PAGE in the Tris-glycine buffer system (A and B) (21), whereas the proteolytic fragments generated from the precursor were analyzed by SDS-PAGE in the Tris-Tricine buffer system (E and F) (22), followed by blotting. HA tag-containing polypeptides on the blots were decorated with the monoclonal antibody against HA (Fig. 3, lanes 4, 5, 7 in αHA). Detection of these polypeptides is described under “Experimental Procedures.” As controls, the samples treated with 0.02 units/μl apyrase for 5 min prior to the docking reaction (Ap; lane 2) or incubated without any energy sources (lane 3) were prepared in the same way as described above. Positions of prSS and mSS are indicated by the filled and the open arrowhead, respectively. In E and G, the same proteolytic fragments as shown in Figs. 1 and 2 are indicated by the open circles denoted as Smaller fragments, whereas the fragments of larger size are indicated by the filled circles denoted as Larger fragments. Intensity of the docked precursor (A and C) and the smaller and the larger fragments (E and G) were quantified as described under “Experimental Procedures.” The ratio of quantified values against the intensity of precursor applied for the docking reaction (A, C, E, and G; lane 1) was calculated and is shown in B, D, F, and H. The images taken from the same blots but from different areas are separated by the dotted vertical lines. N.D., the sample was below detection level. Values represent mean ± S.E.; n = 3–4.

transit peptide was cleaved after translocation, mSS, processed from the precursor biotinylated at position −52, was not detected by streptavidin (Fig. 2A, lanes 4 and 7 in St).

Addition, the amount of the docked precursor (Fig. 3, B and D) and the proteolytic fragments (Fig. 3, F and H) were quantified. The ratio of the quantified value to the value of precursor added
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to the docking reaction (Fig. 3, A, C, E, and G, lane 1) is shown in these panels.

With respect to precursor docking, the amount of the docked precursor was increased more than three times from the basal level when the docking reaction was conducted in the presence of 0.1 mM ATP at 4 °C, regardless of the presence of GTP (Fig. 3, A and B, compare lane 3 with lanes 5, 7, and 8). When ATP concentration was increased from 0.1 to 2.5 mM at 4 °C, the amount of the docked precursor was increased 1.4-fold (Fig. 3, A and B, compare lane 8 with lane 9). Furthermore, when the docking reaction was conducted at 25 °C, more precursor was docked to chloroplasts than at 4 °C under the same NTP conditions (compare Fig. 3, A and B, with Fig. 3, C and D). In the presence of 2.5 mM ATP incubated at 25 °C (Fig. 3C, lane 9), both the chloroplast-docked precursor and imported mSS were observed. When the docking reaction was conducted in the presence of GTP alone, the amount of the docked precursor was almost the same as the basal level (Fig. 3, A and B, lanes 4 and 6; Fig. 3, C and D, lane 4), except in the presence of 2.5 mM GTP incubated at 25 °C. Under this condition, the amount of the docked precursor was approximately twice the basal level (Fig. 3, C and D, lane 6).

After chloroplasts were treated with thermolysin, four proteolytic fragments (open circles) were produced from the precursor docked to chloroplasts in the presence of ATP, irrespective of the incubation temperature (Fig. 3, E and G). Furthermore, when the precursor was docked to chloroplasts in the presence of ATP, three larger proteolytic fragments, with molecular masses between 13.5 and 15 kDa (filled circles), were generated (Fig. 3G, lanes 5 and 7–9), which were also generated from the precursor docked in the presence of 2.5 mM ATP alone at 25 °C but to a lesser extent (Fig. 3G, lane 6). A similar molecular mass range to those of the larger fragments was also produced by trypsin treatment. In addition, the larger fragments, as well as the smaller fragments (Fig. 2B) were localized in the membranes.

We quantified the four smaller fragments (open circles in Fig. 3, E and G) and the three larger fragments (filled circles in Fig. 3, E and G), and the results are shown in Fig. 3, F and H (the amounts of the smaller, and the larger fragments were represented by the open bars and the filled bars, respectively). From this analysis, we concluded that the differences in the proteolytic fragments derived from the precursor are dependent on the degree of translocation through the outer envelope membrane during the docking step (Fig. 3, compare E with G). For instance, when the docking reaction was conducted at 4 °C, approximately half of the docked precursor was completely degradable by thermolysin, whereas the remaining portion of the precursor was partially sensitive, yielding the smaller proteolytic fragments (open circles in Fig. 3E). Conversely, when the docking reaction was conducted at 25 °C, a much lesser amount of the docked precursor was completely degradable by thermolysin. Under these conditions, the majority of the precursor was partially sensitive to protease treatment and degraded to either the smaller or the larger fragments (Fig. 3G, open and filled circles, respectively), indicating that the precursor was sufficiently translocated through the outer envelope membrane. These observations suggest that early intermediates formed under different energy conditions consisted of different combinations of the intermediates. In addition, the observation of the completely degradable precursor docked in the presence of ATP demonstrates the existence of the early intermediate within which the precursor is tightly associated with the outer surface of chloroplasts, although not inserted into the outer envelope membrane, in an energy-dependent manner.

Significance of Internal ATP in the Docking—As seen earlier, different sets of proteolytic fragments were generated from the precursor docked under different temperatures (Fig. 3, E and G). In a previous report, internal ATP was shown to be sufficient for docking (3). To investigate whether a different docking state of the precursor is dependent on internal ATP, the docking reaction was performed with chloroplasts treated in two different ways prior to incubation with the precursor and NTP. (i) Chloroplasts were treated with glycerate to deplete internal ATP (Fig. 4). Nigericin inhibits ATP synthesis in chloroplasts but does not deplete endogenous ATP level, which is lowered by the action of glycerate kinase upon exogenously added glycerate (2). (ii) Chloroplasts were treated with apyrase to deplete external ATP under the dihydroxyacetone phosphate (DHAP) system to generate ATP internally (Fig. 5). The DHAP system consisted of DHAP, oxaloacetate, and P1 (2, 3, 26).

First, using the glycerate treatment, the amount of the precursor docked to chloroplasts in the presence of NTP was reduced to 60–70% but did not reach the basal level (Fig. 4A). After proteolysis, a reduction in the amounts of both the smaller and the larger fragments (open and filled bars, respectively) was observed in the glycerate-pretreated samples (Fig. 4B). These observations indicate that endogenous ATP contributed to the docking, irrespective of exogenously added NTP.

Second, to investigate the role that internal ATP levels play during the docking step, chloroplasts were treated with a combination of apyrase and DHAP before the addition of precursor protein (3) (Fig. 5). Under these treatment conditions, ATP is exclusively generated internally (i.e. within the stroma) (3). As an additional control, regardless of whether ATP and DHAP were added to the docking reactions, all samples presented in Fig. 5 were likewise supplemented with oxaloacetate and P1 (3). This additional treatment alone did not affect the docking step.

When the precursor was incubated with apyrase-treated chloroplasts in the presence of 0.1 mM ATP, the amount of the docked precursor was reduced to the basal level, irrespective of the temperature during the docking reaction (Fig. 5, A and B, lanes 2, 3, 6, and 7). Likewise, after protease treatment, the amounts of both the smaller (open circles and bars) and the larger fragments (filled circles and bars) were also reduced during apyrase treatment (Fig. 5, C and D, compare lanes 2, 3, 6, and 7). We conclude from this result that when externally supplied ATP is sufficiently depleted by apyrase treatment, the engagement of the precursor protein with the outer envelope membrane during the docking step is significantly altered.

However, when the docking reaction was conducted at 4 °C in the presence of both apyrase and DHAP, conditions that exclusively generate ATP internally, the amount of precursor
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that docked to chloroplasts was almost the same as when precursor was docked in the presence of 0.1 mM ATP alone without apyrase treatment (Fig. 5, A and B, compare lane 2 with lanes 4 and 5). Furthermore, the same amount of the small proteolytic fragments (open circles and bars) was produced from either of these treatment conditions (Fig. 5, C and D, compare lanes 2, 4, and 5). Hence, we conclude that it is the internal ATP levels that play a critical role during the docking step.

Finally, when the docking reaction was conducted at 25 °C, in the presence of both apyrase and DHAP, the amount of docked precursor was reduced by ~70% when compared with the amount of precursor docked in the presence of 0.1 mM ATP without apyrase treatment (Fig. 5, A and B, compare lane 6 with lane 9). We conclude that the majority of this reduction can be attributed to the docked precursor that has been converted to the larger proteolytic fragment (filled circles and bars) after protease treatment (Fig. 5, C and D, compare lane 6 with lane 9). Additionally, as shown in a previous study (3), a small portion of the docked precursor may likewise have been reduced due to some of the docked precursor protein being importedit and processed to a mature form when incubated at 25 °C (Fig. 5A, lanes 8 and 9). Nevertheless, taken together, the results presented here (Fig. 5) indicate that internally generated ATP is sufficient to support both the formation of early intermediates and for the complete translocation of the precursor protein into chloroplasts even in the absence of “externally” supplied ATP.

Transition between Docking States—In the presence of 0.1 mM ATP, it is suggested that more precursor is translocated deeper at 25 °C than at 4 °C. As shown in Fig. 2A, the precursor docked to chloroplasts at 4 °C was processed to the mature form when it was chased in the presence of 2.5 mM ATP at 25 °C. We wondered if the precursor docked to chloroplasts at 4 °C would show further penetration when the temperature was increased from 4 to 25 °C. After the docking reaction was performed at 4 °C in the presence of 0.1 mM ATP (Fig. 6, lane 2), followed by the reisolation of chloroplasts, the docked precursor was chased by incubating chloroplasts at 4 or 25 °C in the presence of apyrase or 0.1 mM ATP or 2.5 mM ATP (Fig. 6, lanes 3–8). Apyrase was added as a control (Fig. 6, lanes 3 and 6).

The amount of the docked precursor was not affected by the chase except when 2.5 mM ATP was present at 25 °C during the chase (Fig. 6, A and B), when the precursor was processed into the mature form, as shown in Fig. 2A. After proteolysis, the smaller fragments (open circles and bars) were decreased, and whereas the proteolytic fragments generated from the precursor were analyzed by SDS-PAGE in the Tris-Tricine buffer system (A) (21),

FIGURE 4. Docking is inhibited by depleting the internal ATP. After nigericin-pretreated chloroplasts were incubated for 5 min either in the presence or in the absence of 1 mM glycerate at the same temperature as the following reaction, biotinylated prSSC1(1–52)HAHAH was added, and the docking reaction was performed under various conditions for 5 min, followed by the thermolysin (0.8 mg/mg of chlorophyll) treatment at 4 °C for 30 min. The docked precursor was analyzed by SDS-PAGE in the Tris-glycine buffer system (A) (21),
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FIGURE 5. Early intermediates are formed only by the internal ATP. After chloroplasts, pretreated with nigericin, were incubated for 5 min with 20 mM Na-Pi buffer, pH 8.0, and 1 mM oxaloacetate either in the presence or in the absence of 0.02 units/μl apyrase, biotinylated prSSC1(−52)HAHAHA was added, and the docking reaction was performed in the presence of 0.1 mM ATP or 0.1 mM DHAP at 4 or 25 °C for 5 min, followed by thermolysis (0.8 mg/mg of chlorophyll) treatment at 4 °C for 30 min. The docked precursor was analyzed by SDS-PAGE in the Tris-glycine buffer system (A) (21), whereas the proteolytic fragments generated from the precursor were analyzed by SDS-PAGE in the Tris-Tricine buffer system (C) (22), followed by the blotting. HA tag-containing polypeptides on the blots were decorated with the mouse monoclonal antibody against HA (αHA); whereas biotinylated polypeptides were decorated with streptavidin (St). Detections of these polypeptides are described under “Experimental Procedures.” In A and C, positions of prSS and mSS are indicated by the filled circles and bars, respectively. The protelytic fragments (C) are indicated by the open circles denoted as Smaller fragments (D), whereas the fragments of larger size (C) are indicated by the filled circles denoted as Larger fragments (D). Intensity of the docked precursor and the smaller and the larger fragments was quantified as described under “Experimental Procedures,” and the ratios of the quantified value against the intensity of the precursor applied for the docking reaction (A and C, lane 1) were calculated and are shown in B and D. The images taken from the same blots but from the different areas are separated by the dotted vertical lines. N.D., the sample was below the detection level. Values represent mean ± S.E.; n = 3.

From these results, it can be concluded that the movement of the precursor within the early intermediate could be chased, and this transition required a low concentration of ATP and increased temperature. These results suggest that there are at least two individual ATP-requiring steps in the early stage of import, one of which is temperature-sensitive and the other temperature-insensitive.

Importance of ATP or GTP Hydrolysis in Precursor Docking—
Two Toc components, Toc159 and Toc34, possess a GTP-binding domain and hydrolyze GTP (27). Moreover, Hsp70 is reported to be present at the intermembrane space, which associates with the precursor during protein translocation (10). The requirement of GTP or ATP at the docking reaction may be related to these components. Hence, the question arises whether hydrolysis of GTP or ATP is essential for the formation of the early intermediate. Olsen et al. (2) have reported that docking at room temperature required ATP hydrolysis. On the other hand, Young et al. (4) have reported that GTP hydrolysis is important for the association of precursors to chloroplasts and that the GTP-requiring step was ahead of the ATP-requiring step.

In order to determine if docking at 4 °C requires hydrolysis of NTP, the docking reaction was initiated in the presence of the slowly hydrolyzable analogues of GTP and ATP, GTPγS, and ATPγS, respectively (Fig. 7, A and B). In the presence of ATPγS, the docked precursor remained at the basal level irrespective of temperature during the docking reaction, indicating that ATPγS was unable to support docking observed in the presence of ATP (Fig. 7, A and B, lanes 5 and 9). On the other hand, ATP support docking was inhibited when GTPγS and ATP coexisted during the reaction (Fig. 7, A and B, lanes 4 and 8). After proteinolysis, the amount of the smaller (open circles and bars) and the larger fragments (filled circles and bars) were significantly increased if the chase was performed in the presence of 0.1 mM ATP at 25 °C but not at 4 °C (Fig. 6, C and D). We also conducted the chase experiments of the precursor docked at 25 °C in the presence of 0.1 mM ATP by decreasing the temperature to 4 °C in the presence of 2.5 mM ATP. The amount of the docked precursor and both the smaller and the larger fragments was not changed by lowering the temperature.ATP or 0.1 mM DHAP at 4 or 25 °C for 5 min, followed by thermolysis (0.8 mg/mg of chlorophyll) treatment at 4 °C for 30 min. The docked precursor was analyzed by SDS-PAGE in the Tris-glycine buffer system (A) (21), whereas the proteolytic fragments generated from the precursor were analyzed by SDS-PAGE in the Tris-Tricine buffer system (C) (22), followed by the blotting. HA tag-containing polypeptides on the blots were decorated with the mouse monoclonal antibody against HA (αHA); whereas biotinylated polypeptides were decorated with streptavidin (St). Detections of these polypeptides are described under “Experimental Procedures.” In A and C, positions of prSS and mSS are indicated by the filled circles and bars, respectively. The protelytic fragments (C) are indicated by the open circles denoted as Smaller fragments (D), whereas the fragments of larger size (C) are indicated by the filled circles denoted as Larger fragments (D). Intensity of the docked precursor and the smaller and the larger fragments was quantified as described under “Experimental Procedures,” and the ratios of the quantified value against the intensity of the precursor applied for the docking reaction (A and C, lane 1) were calculated and are shown in B and D. The images taken from the same blots but from the different areas are separated by the dotted vertical lines. N.D., the sample was below the detection level. Values represent mean ± S.E.; n = 3.
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**DISCUSSION**

Under restricted energy conditions, the precursor destined to chloroplasts binds irreversibly, “docking” to form the early translocation intermediate. Although the docking step has only been observed in the *in vitro* protein import assay, it is regarded as an important step in the early stage of import, mediating between precursor recognition at the surface of chloroplasts and precursor translocation through the Toc and Tic complexes. Many Toc and Tic components have been found and identified by analyzing the early translocation intermediates (10, 12–16). Previous studies on the early translocation intermediate have focused on the conditions required for the formation of intermediates (2–4, 23). However, the behavior of the precursor in the early intermediate has not been examined. In the present study, the early intermediate formed under different conditions has been evaluated by analyzing the extent of translocation of the precursor in the early intermediate. We have established an assay system where the early intermediate was formed under stringent energy conditions and the location of precursors within the early intermediate by limited proteolysis was determined. We were also able to analyze the docking step as an event solely between the precursor and chloroplasts, since a purified recombinant precursor was used in the assay system, thereby avoiding any factors that would affect the docking reaction and that may be present had the precursor been synthesized using the *in vitro* transcription-translation system (28).

Analysis of the proteolytic fragments suggests that the docking step had at least three steps based on the GTP/ATP requirement and temperature (Fig. 8). Each individual step consisted of a different set of intermediates containing different states of the precursor. Three states of the precursor associated with chloroplasts have been observed. These have been identified based on the size of proteolytic fragments generated from the precursor as the “energy-dependent association,” “insertion,” and "docking reaction and that may be present had the precursor been synthesized using the "in vitro" transcription-translation system (28).

Intensity of the docked precursor and the smaller and the larger fragments was quantified as described under “Experimental Procedures,” and the ratios of the quantified value against the intensity of the precursor were applied for the docking reaction (A and C, lane 1) were calculated and shown in B and D. The images taken from the same blots but from different areas were separated by dotted vertical lines. Values represent mean ± S.E.; n = 3.
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A–D, the docking reaction was performed by incubating nigericin-pretreated chloroplasts with biotinylated prSSC1(−52)HAHAH for 5 min under the different conditions indicated in the figure. Chloroplasts were treated with 0.8 mg of thermolysin/mg of chlorophyll at 4 °C for 30 min. E–H, after the docking reaction was performed by incubating nigericin-pretreated chloroplasts with biotinylated prSSC1(−52)HAHAH in the presence of 0.1 mM ATP at 4 °C for 5 min, intact chloroplasts were recovered through a 40% Percoll cushion. Chloroplasts were resuspended and incubated at 25 °C for 5 min in the presence of either 0.1 mM ATP or 0.1 mM ATP/5S. The docked precursor were analyzed by SDS-PAGE in the Tris-glycine buffer system (A and E) (21), whereas the proteolytic fragments generated from the precursor were analyzed by SDS-PAGE in the Tris-Tricine buffer system (C and G) (22), followed by the blotting. HA tag-containing polypeptides on the blots were decorated with the monoclonal antibody against HA (αHA; A and E), whereas biotinylated polypeptides were decorated with streptavidin (St; C and G). Detection of these polypeptides were described under “Experimental Procedures.” In A, C, E, and G, the positions of prSS and mSS are indicated by the filled and the open arrowhead, respectively. The proteolytic fragments (D and H) are indicated by the open circles denoted as Smaller fragments (C and G), whereas the fragments of larger size (C and G) are indicated by the filled circles denoted as Larger fragments (D and H). Intensity of the docked precursor and the smaller and the larger fragments was quantified as described under “Experimental Procedures,” and the ratios of the quantified value against the intensity of the precursor applied for the docking reaction (A, C, E, and G, lane 1) were calculated and shown in B, D, F, and H. The images taken from the same blots but from the different areas are separated by the dotted vertical lines. N.D., the sample was below the detection level. Values represent mean ± S.E.; n = 3.

“penetration” states. In the “energy-dependent association” state, the precursor is associated with chloroplasts yet digested completely. “Insertion” is represented by the four fragments between 7.8 and 12 kDa, and “penetration” is represented by the three larger fragments between 13.5 and 15 kDa. Because precursor docked to chloroplasts is thought to be unfolded, bound precursor might be digested at multiple sites where proteases allowed their high accessibility. ATP hydrolysis played an important role in the transition between the states (Fig. 7), and each state of the precursor could be chased by altering the experimental conditions (Fig. 6). When the precursor was incubated with chloroplasts in the presence of ATP at 4 °C, the majority of the docked precursor was in the ‘energy-dependent association’ and ‘insertion’ states (Figs. 1–3). On the other hand, in the presence of ATP at 25 °C, ‘penetration’ and ‘insertion’ were the most common states of the precursor (Fig. 3). Transition observed by shifting the temperature from 4 to 25 °C was irreversible. Both the larger and smaller proteolytic fragments were observed when the precursor was docked to chloroplasts at 25 °C and then the temperature was lowered to 4 °C.  

Two important conclusions can be drawn from the proteolytic fragments. First, whether the docking reaction was performed at 4 or 25 °C, the entire length of the transit peptide of prSS was inserted into the outer envelope membrane. Similar sized fragments were observed when proteolytic fragments were detected through biotin-modified precursor carrying a single cysteine residue at position −52, −33, or +4. This is the first report of the transit peptide being penetrating into the outer envelope membrane at 4 °C. Second, the precursor penetrated further into the membrane when the docking reaction was performed at 25 °C than at 4 °C. Based on the molecular mass of the fragments, the smaller fragments or the larger fragments may consist of the entire transit peptide (57 amino acid residues) and 20–55 or 65–80 amino acid residues of the amino-terminal part of mSS, respectively. These conclusions are partly consistent with the report by Waegemann and Soll (17), in which they also proteolyzed the precursor after incubation with chloroplasts in the presence of 0.1 mM ATP at 25 °C and obtained four fragments. On the other hand, they did not observe any proteolytic fragments at 4 °C. It is highly possible that these fragments are the same as the larger fragments observed in this study. However, unlike this study, they did not use controls to determine the precise part of the precursor that produced the proteolytic fragments or assess the accessibility of proteases to the outer surface of the chloroplasts.

Then how does temperature affect the docking reaction? It is well known that temperature change causes phase transition in lipids. Little change in membrane fluidity has been detected between 4 and 25 °C (5). It was concluded by these authors that the low activity of the ATP/ADP carrier protein caused import inhibition at low temperature. However, this is not the case for docking, because internal ATP generated by the DHAP system (3, 26) supported precursor docking to chloroplasts at the same level as externally supplied ATP (Fig. 5). ATP hydrolysis was essential for the formation of the intermediate both at 4 °C and at 25 °C as well as the transition from the docking state at 4 °C to the docking state at 25 °C (Fig. 7). These results indicate that there are two independent ATP hydrolysis steps for docking,
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FIGURE 8. A working model for the early stage of protein import into chloroplasts. Based on the observations from the present study and a previous study (4), a model for the early stage of import into chloroplasts is proposed. Three different types of early translocation intermediate (I, II, and III) are formed at the docking step under different energy conditions. In the bottom part of the figure, the wide arrow in the solid line shows the extent to which the precursor proceeded under each docking condition, whereas the arrow in the dotted line indicates the possible extent to which the precursor proceeded. OE and IE, the outer and the inner envelope membranes, respectively.

one of which is temperature-sensitive. No ATPase other than HSP70-IAP (10) has been identified to be involved in the early stage of protein import. Although no direct evidence is present, HSP70-IAP may be involved in both ATP hydrolysis steps or may be involved in only one of the steps, and an unidentified protein may be involved in the other step.

Toc core complex contains two receptor components of Toc159 and Toc34, both of which have a GTPase domain (27). GTP has been shown to support precursor binding to chloroplasts (3), whereas GTPase activity of these Toc components has been stimulated by the precursor (29–31). The role of GTP and GTP hydrolysis in protein import into chloroplasts has been hypothesized in the two models: the targeting hypothesis and the motor hypothesis (1, 9). In the targeting hypothesis, Toc159 is the primary receptor for precursors, cooperating with Toc34 to promote transfer of precursors to Toc75 by GTP hydrolysis (1, 9), whereas in the motor hypothesis, Toc34 is the primary receptor for precursors and Toc159 might drive protein translocation through the outer envelope membrane by GTP hydrolysis from the analysis of the reconstituted Toc core complex into proteoliposomes (30). Furthermore, Young et al. (4) attempted to determine the step at which GTP was involved in protein import into chloroplasts by analyzing the precursor associated with chloroplasts. They concluded that GTP mediated the steps between energy-independent binding and the formation of ATP-dependent docking and was not involved in protein translocation. Based on these reports, we attempted to investigate the role of GTP under stringent energy conditions by applying our assay system.

When the docking reaction was conducted in the presence of GTP as a sole NTP, the amount of the precursor docked to chloroplasts was increased ~2-fold from the basal level only when 2.5 mM GTP was present at 25 °C (Fig. 3). This result is consistent with the report of Young et al. (4). However, there is a possibility that the residual internal ATP affected precursor binding in the presence of GTP. Therefore, the internal ATP level was reduced by adding glycerate before the docking reaction in the presence of GTP at 25 °C (Fig. 4). The amount of the docked precursor was reduced to ~60% by adding glycerate, which indicates that the residual endogenous ATP does contribute to docking. However, despite depleting the internal ATP, a considerable amount of the precursor was still bound to chloroplasts. Therefore, GTP may have two possible roles; GTP may be involved in docking either directly or indirectly.

Precursor docking to chloroplasts was inhibited by GTPγS even when 0.1 mM ATP was present (Fig. 7 in this report) (4), which indicated the direct involvement of GTP.

Chloroplasts contain nucleoside diphosphate kinase, which catalyzes the transfer of γ-P, from NTP to NDP of other nucleosides (32). Therefore, conversion from GTP to ATP in chloroplasts is highly possible, which may support docking. In addition, more precursor in the “penetration” state was found when incubated with 2.5 mM GTP at 25 °C than with 0.1 mM ATP at 4 °C. These results indicate that ATP converted from GTP, rather than GTP by itself, contributed to the precursor reaching the “penetration” state. On the other hand, docking observed in the presence of ATP alone can also be explained by the action of nucleoside diphosphate kinase, converting ATP to GTP. Taken together, GTP may involve in docking directly in the earlier stage and indirectly in the later stage. Furthermore, “energy-dependent association” may contain both GTP-dependent association and ATP-dependent association (Fig. 8). Although we are unable to conclude which energy-dependent association precedes the other, Young et al. (4) have reported that the GTP-requiring step was ahead of the ATP-requiring step (Fig. 8).

As shown in Fig. 3, we did not observe an enhancement of ATP-dependent docking by GTP as reported by Young et al. (4), possibly due to the difference in precursor preparations. The in vitro transcription/translation mixture used by those authors to obtain the precursor (4) might have additional factor(s) that enhance ATP-dependent docking in the presence of GTP. Although there are several controversies (1, 9), the observation of the residual GTP-dependent association by the authors does not necessarily rule out the GTP-driven motor hypothesis of Schleiff et al. (30) during the early stage of docking. The possible blockage of precursor translocation through the outer envelope membrane by a factor at the intermembrane space or the inner envelope membrane did not occur in their reconstituted system. Further investigations are required to...
understand precisely how GTP and its hydrolysis are involved in protein import.

Analysis of the size of proteolytic fragments raises the question whether it is the insertion state or the penetration state of the precursor that is inserted into the inner envelope membrane. As described earlier, sufficient length of the precursor for insertion into the inner envelope membrane is translocated through the outer envelope membrane. In the present study, the early intermediate focusing solely on the state of the precursor was analyzed and not the state of the translocon under different docking conditions; therefore, it is difficult to answer this question. However, earlier cross-linking studies give some insights into the state of early intermediates associated with precursors. Tic22 and Tic20 cross-linked to the precursor when the docking reaction was performed at 26 °C (15) but not at 4 °C (12). Chen and Li (33) showed the presence of at least two translocon complexes that were associated with precursors during active import by analyzing the cross-linked intermediate by blue native PAGE. In addition, Akita et al. (14) reported multiple translocon complexes cross-linked with precursor, although the early intermediate was formed in the presence of ATP at 4 °C. Furthermore, some of the cross-linked products contained Tic110 and Hsp93 in the stroma (14). It is possible that these complexes are the same early intermediates that were observed in this study. Other site-specific cross-linking experiments by using single cysteine precursors to analyze the early-intermediate are currently under way, which may help answer this question.

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