Published in final edited form as:

Methods Mol Biol. 2011 ; 774: 307–320. doi:10.1007/978-1-61779-234-2_18.

Energetic Manipulation of Chloroplast Protein Import and the Use of Chemical Cross-Linkers to Map Protein–Protein Interactions

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Abstract

Most chloroplast proteins are synthesized in the cytosol as preproteins with N-terminal cleavable transit peptides and are imported into the organelle through the TOC–TIC translocon system. Import involves a complex set of recognition and membrane translocation steps that ensure the fidelity and unidirectional transport of the polypeptide across the double-membrane chloroplast envelope. To understand the mechanism of import, the molecular interactions and energetics of each step must be defined. Here, we describe the methods for capturing intermediates in the import process through the manipulation of the energy state of chloroplasts, and the use of two different chemical cross-linking approaches to examine the molecular interactions that mediate the import process and to assess the assembly state of the translocons. These approaches can be employed to identify sequential protein–protein interactions, and thereby dissect the pathway and roles of import components during protein import into chloroplasts.

Keywords

Chloroplast; Import; TOC; TIC; GTP; ATP; Cross-link; Early import intermediate

1. Introduction

The biogenesis of chloroplasts relies on the import of thousands of nucleus-encoded proteins from the cytoplasm (1). Most chloroplast proteins are synthesized as preproteins with N-terminal cleavable transit peptides. Multimeric translocons embedded in the outer (TOC) and inner (TIC) envelope membranes (2, 3) mediate the recognition of preproteins at the chloroplast surface and their translocation across double-membrane envelope. The core of the TOC translocon consists of two receptor GTPases, Toc159 and Toc34, which associate with a membrane channel Toc75. The TOC receptors are required for transit peptide recognition at the organelle surface, and they initiate the import process by transferring the preprotein to the protein-conducting channel. Components of the TIC complex (Tic20/21, Tic22, and Tic110) associate with the early import intermediates that are inserted into the TOC channel (4–7), and inner membrane translocation is powered by a proposed chaperone motor complex, containing Tic40, Hsp93, and Hsp70, at the trans side of the inner envelope membrane (8, 9).

A number of discernable steps in import have been defined by manipulating the temperature and energy state of chloroplasts during in vitro protein import reactions. The most detailed analysis of the energetics of import and the corresponding intermediates in preprotein translocation has been performed with pea chloroplasts (10). Although these intermediates likely represent basic mechanistic steps that also apply to Arabidopsis, only four discernable steps in import have been examined in Arabidopsis chloroplasts. These include (1) an initial, reversible binding step that is observed in the absence of NTPs and corresponds to the first interaction of the transit peptide with the TOC translocon, (2) a GTP-stimulated binding that has been observed using TOC receptor GTPase mutants, (3) ATP-dependent insertion of the preprotein into the TOC channel (early import intermediate), and (4) subsequent ATP-driven translocation across the inner membrane via the TIC channel and associated molecular chaperones.

Although the core components of the TOC and TIC translocons and specific steps in import have been identified (5, 11, 12), defining the molecular mechanism of import requires knowledge of the molecular interactions between the preprotein and import components at each stage. Key questions include the following: (1) what is the molecular basis of transit peptide recognition by the TOC receptors, (2) how do the intrinsic GTPase cycles of the receptors control the transition from initial binding to membrane translocation and thereby regulate the fidelity of import, (3) what is molecular nature of each membrane channel and how are they coupled to provide a continuous translocation pathway, and (4) how is the system of molecular chaperones that associates with the import apparatus organized to provide the unidirectional driving force for translocation?

The availability of well-defined import intermediates, coupled with the ability to generate specific mutants in Arabidopsis, now provides a powerful system to gain insight into the molecular details of the import reaction. Recently, we generated Arabidopsis transgenic plants expressing a mutated form of Toc159 with significantly reduced GTPase activity to examine the role of receptor GTPase activity in protein import (13). To characterize the state of import intermediates in the GTPase mutant, we developed chemical cross-link approaches to map preprotein–translocon interactions (13). In a second approach, we used an independent covalent cross-linking approach to determine the effects of mutations in Tic110 on the dynamic assembly of protein import complexes (4). In this chapter, we will focus on methods that couple the generation of import intermediates in wild-type and mutant chloroplasts with covalent cross-linking to examine the dynamic assembly of import complexes and identify the roles of core TOC/TIC components at each stage in import.

2. Materials

2.1. Isolation of Chloroplasts from *Arabidopsis thaliana*

1. MS plates: 2-(4-morpholino)ethane sulfonic acid (MES)-KOH, pH 5.7, 0.5× Murashige and Skoog (MS) growth medium, 1% (w/v) sucrose, and 0.8% (w/v) Phytoagar.
2. 150 × 15 mm Petri plates (Falcon).
3. Percoll (GE Healthcare).

4. Miracloth (Calbiochem).
5. Protease Inhibitor Cocktail P9599 (PIC) (Sigma–Aldrich).
6. Ultra-Turrax T 25 (IKA Labortechnik) with a 15-mm probe (or comparable). This is a handheld rotor-stator homogenizer. Similar models produced by Polytron can also be used.
7. Grinding buffer (GB): 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES)-KOH, pH 7.5, 330 mM sorbitol, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM MnCl₂, and 1 mM MgCl₂. The following reagents are added immediately prior to use: 100 mM ascorbic acid, 0.25% (w/v) bovine serum albumin (BSA), and 0.05% (v/v) PIC.
8. 40% Percoll: 40% (v/v) Percoll, 50 mM HEPES-KOH, pH 7.5, 330 mM sorbitol, 2 mM EDTA, 1 mM MnCl₂, and 1 mM MgCl₂. Store at –20°C. The following reagents are added immediately prior to use: 0.25% (w/v) BSA, 100 mM ascorbic acid, and 0.05% (v/v) PIC.
9. 85% Percoll: 85% (v/v) Percoll, 50 mM HEPES-KOH, pH 7.5, 330 mM sorbitol, 2 mM EDTA, 1 mM MnCl₂, and 1 mM MgCl₂. Store at –20°C. The following reagents are added immediately prior to use: 0.25% (w/v) BSA, 100 mM ascorbic acid, and 0.05% (v/v) PIC.
10. Two Percoll step gradients: 1 mL of 85% Percoll overlaid with 4 mL of 40% Percoll in 15 round-bottomed 15-mL glass Corex centrifuge tubes.
11. HS buffer: 50 mM HEPES-KOH, pH 7.5, and 330 mM sorbitol. Store at –20°C.
12. 80% (v/v) acetone.
13. 250-mL Nalgene polypropylene screw-capped centrifuge bottles.
14. Sorvall RC-5 superspeed centrifuge or comparable.
15. Sorvall GSA fixed-angle and HB-4 swinging bucket centrifuge rotors or comparable.
16. Microcentrifuge.
17. Vortex mixer.

2.2. Energy Depletion of Isolated Chloroplasts

1. Nigericin (Calbiochem). This is dissolved in 80% (v/v) ethanol to a concentration of 40 mM. Store at –20°C.
2. Apyrase (Sigma–Aldrich). This is dissolved in HS buffer to a concentration of 100 u/μL (units/microliter). Store at –80°C.
3. Glycerate (Sigma–Aldrich). This is dissolved in HS buffer to a concentration of 100 mM immediately prior to use.

2.3. Preparation of preSSU by Coupled In Vitro Transcription/Translation

1. The cDNA encoding the precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (preSSU) from Arabidopsis (At5g38430) cloned downstream of the T7 polymerase promoter in the pET21a plasmid.
2. TNT-coupled reticulocyte lysate system (Promega).
3. 10 $\mu\text{Ci}/\mu\text{L}$ [^{35}S]-methionine (Perkin Elmer).
4. 40 u/ μL (units/microliter) RNase inhibitor (New England Biolabs).
5. Hydrated Sephadex G-25 (Pharmacia Biotech Inc.). This is equilibrated with HS buffer prior to use.

2.4. Generation of Protein Import Intermediates and Chemical Cross-Linking to Map Translocon–Preprotein Interactions

1. 10 $\times$ MMX (chloroplast import master mix): 250 mM potassium acetate (KOAc) and 50 mM magnesium acetate (MgOAc). Store at -20°C .
2. 35% Percoll: 35% (v/v) Percoll, 330 mM sorbitol, and 50 mM HEPES-KOH, pH 7.5. Store at -20°C .
3. ATP-NaOH, pH 7.5, is dissolved in HS buffer to a final concentration of 100 mM and stored at -20°C .
4. GTP-NaOH, pH 7.5, is dissolved in HS buffer to a final concentration of 100 mM and stored at -20°C .
5. In vitro-translated [^{35}S]preSSU (see Subheading 3.3).
6. SDS-PAGE sample buffer: 350 mM Tris base, 5% (w/v) sodium dodecyl sulfate (SDS), 80 mM dithiothreitol (DTT), 7.5% (v/v) glycerol, and 0.1% saturated bromophenol blue. Prepare immediately prior to use.
7. Dithio-bismaleimidoethane (DTME, Thermo Fisher Scientific). This is dissolved in dimethylsulfoxide (DMSO) to a final concentration of 50 mM immediately prior to use.
8. 2% (w/v) SDS.
9. Dilution buffer: 50 mM Tris-HCl, pH 7.5, 1.1% (w/v) Triton X-100, 167 mM NaCl, 5.6 mM EDTA, and 1% (v/v) PIC. Prepare immediately prior to use.
10. Protein-A Sepharose CL-4B (GE Healthcare) is equilibrated with dilution buffer prior to use.
11. Washing buffer A: 50 mM HEPES-KOH, pH 7.5, 0.1% (w/v) Triton X-100, 150 mM NaCl, and 0.1% (v/v) PIC. Prepare immediately prior to use.
12. Microcentrifuge.
13. Phosphorimager.

2.5. Chemical Cross-Linking to Map Translocon Assembly and Dynamics

1. 50 mM dithiobis[succinimidyl propionate] (DSP) in DMSO (Thermo Fisher Scientific). Prepare immediately prior to use.
2. TES buffer: 50 mM N-[Tris(hydroxymethyl)methyl]glycine (Tricine)-KOH, pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% (w/v) Triton X-100, and 1% (v/v) PIC. Prepare immediately prior to use.
3. Protein-A Sepharose CL-4B (GE Healthcare) equilibrated with TES buffer. Prepare immediately prior to use.
4. Washing buffer B: 50 mM Tricine-KOH, pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.1% (w/v) Triton X-100, and 0.1% PIC. Prepare immediately prior to use.
5. Microcentrifuge.

3. Methods

3.1. Isolation of Chloroplasts from *Arabidopsis thaliana*

Traditionally, pea chloroplasts have been used as a model system for assaying chloroplast preprotein import. Peas offer the advantages of large amounts of material that can be grown quickly and easily, and rapid and simple isolation of intact, import-competent chloroplasts (14, 15). However, the tools available in *Arabidopsis thaliana* for the investigation of genetic, biochemical, and cellular processes make it desirable to use this model system to analyze chloroplast targeting (4, 16, 17). The small size of *Arabidopsis* and the need to use young rapidly growing tissues for chloroplast isolation present challenges in adapting the *Arabidopsis* system for the in vitro analysis of chloroplast import. A number of methods for chloroplast isolation from *Arabidopsis* have been published, but many of them require expensive and time-consuming methods, or result in chloroplasts with low levels of import activity. We have found that the following method adapted from two previously published procedures (18, 19) provides excellent yields of highly import-active chloroplasts (13). This method also significantly reduces degradation of the Toc159, a common problem with other methods used for isolation of *Arabidopsis* and pea chloroplasts.

1. Spread sterilized *Arabidopsis* seeds on MS plates (0.1 g seeds per plate) and incubate the plates at 4°C for 24–48 h. Transfer the plates to a growth chamber (16-h day period, 22°C) and allow growth for 14 days (see Note 1).
2. Harvest plants by shaving the green shoots from the surface of the agar plates with a razor and place the shoots directly in 100 mL of ice-cold GB. Plants harvested from up to 2 plates (~10 g plant material wet weight) can be added to 100 mL of GB (see Note 2).

¹Although chloroplasts can be isolated from older plants to provide higher yields, the protein import activity decreases with the age of the plants. We have found that 14-day-old plants grown under these conditions yield reasonable amounts of highly import-active chloroplasts.

²Temperature and the time of isolation are the most important factors that will determine the quality of the isolated chloroplasts. It is essential that all reagents for the isolation be kept ice-cold and that all procedures are performed on ice or in a cold room. The chloroplast isolation procedure should not take >1 h from the time of tissue homogenization to isolation of the intact chloroplasts.

3. Homogenize the plant suspension for <1 min using a Ultra-Turrax T25 rotor-stator homogenizer with a 15-mm probe (or comparable) at 8,000 rpm.
4. Filter the solution by gravity through two layers of Miracloth into a 250-mL centrifuge bottle held on ice.
5. Centrifuge the solution for 8 min at $1,000 \times g$ at 4°C in a Sorvall GSA fixed-angle rotor. Decant the supernatant and discard, taking care to leave the loose pellet behind.
6. Resuspend the pellet by slow swirling in 10 mL of fresh GB. Ensure that the suspension remains cold and that no large clumps remain. Do not shake or introduce air bubbles.
7. Layer one-half of the suspension onto one each of two Percoll step gradients. Use a large bore pipette to transfer all solutions containing plant extracts to avoid sheering the chloroplasts (see Note 3).
8. Centrifuge the gradients at $7,700 \times g$ for 15 min at 4°C in an Sorvall HB-4 rotor using slow acceleration and deceleration (see Note 4).
9. Remove and discard the GB and the 40% Percoll layer by aspirating from the top of the gradient. Be careful not to disturb the 40–85% Percoll interface. This layer contains the intact chloroplasts. Collect the intact chloroplasts at the 40–85% Percoll interface by slow pipetting and transfer into 10 mL of cold HS buffer in a 15-mL glass Corex centrifuge tube. The layer of chloroplasts can be somewhat viscous. The chloroplasts can be washed out of the pipette by very slowly pipetting up and down with HS buffer.
10. Mix the solution by covering the tube with Parafilm and gently inverting twice. Centrifuge the suspension at $1,000 \times g$ for 6 min at 4°C in an Sorvall HB-4 swinging bucket rotor. Carefully discard the supernatant. Take care not to pour off the pellet because the pellet is very loose.
11. Completely resuspend the pellet by gentle swirling with 400 µL of cold HS buffer. Do not shake vigorously or use a pipette.
12. Quantify the yield of chloroplasts by measuring the chlorophyll content. Dilute 5 µL of the chloroplast suspension into 995 µL of 80% acetone. Mix the acetone solution vigorously using a vortex mixer and centrifuge for 1 min at maximum speed at room temperature in a microcentrifuge to remove cell debris. The absorbance of the supernatant is measured at 652 nm and chlorophyll content measured by the following formula: Chlorophyll (in mg/mL) is calculated as

³High acceleration/deceleration rates disrupt step gradients. After centrifugation of the Percoll step gradients, two distinct bands of very dark green material will be obvious at the GB–40% Percoll interface (broken chloroplasts and cell debris) and the 40–85% Percoll interface (intact chloroplasts).

⁴Larger volume gradients can be used if more than five plates of plants are to be processed. This will give a better resolution of fractions on the step gradients. To scale up the Percoll gradients, layer 10 mL of 40% Percoll over 5 mL of 85% Percoll in a 30-mL round-bottomed centrifuge tube (e.g., Corex 30-mL centrifuge tube). The gradients can be prepared in advance and kept on ice before harvesting the plants.

$(A_{652}/36) \times 200$ (dilution factor). Dilution factor = total volume/volume sample added.

13. Once the chlorophyll content has been determined, the purified chloroplasts are diluted to a final concentration of 1 mg chlorophyll/mL with HS buffer and kept on ice in a covered container until use (see Note 5).

3.2. Energy Depletion of Isolated Chloroplasts

To control the energy state of in vitro import reactions, it is essential to deplete chloroplasts of endogenous ATP pools, preclude light-driven ATP synthesis, and remove external GTP. In general, we have found incubation of isolated chloroplasts in the dark at room temperature for a short period sufficient to deplete endogenous ATP for assays to generate early import intermediates. In cases where it is desirable to examine very detailed steps in import (10, 20), chloroplasts are further treated with nigericin, an ionophore that dissipates the light-generated proton gradient at thylakoid membranes (21), and with glycerate to ensure the depletion of existing stromal ATP. Glycerate is the substrate for glycerate kinase (22, 23). To examine energy-independent binding of preproteins to TOC translocons, apyrase is added to degrade external NTPs to NDPs and NMPs (23).

1. Isolated chloroplasts (see Subheading 3.1) are incubated in the dark for 10 min at 23–26°C to deplete internal ATP prior to use in the generation of all import intermediates.
2. To maximize ATP depletion of isolated chloroplasts (see Subheading 3.1), glycerate is added to a final concentration of 10 mM (22) and nigericin to a final concentration of 400 nM prior to the incubation in step 1 above.
3. If chloroplasts are to be used to measure energy-independent preprotein binding, apyrase (2 units/100 µL) is added to the isolated chloroplasts prior to the incubation in step 1 above.

3.3. Preparation of preSSU by Coupled Transcription-Translation

[³⁵S]-methionine-labeled in vitro-translated preproteins are synthesized using cDNAs cloned into plasmids downstream of a T7 RNA polymerase promoter. The pET21 series of vectors are convenient as they can be used for both in vitro translation and *E. coli* expression. A commercially available reticulocyte lysate system (Promega) allows the in vitro synthesis of the protein by coupling transcription and translation. The reaction is carried out in the presence of a radioactive [³⁵S] methionine in order to label the newly synthesized protein for detection by phosphorimaging or autoradiography. Both the wheat germ and the rabbit reticulocyte lysate systems have been used for protein synthesis and import into chloroplasts. We routinely use the rabbit reticulocyte lysate system because the yield and quality of the translation products exceed those synthesized using the wheat germ system. The protocol described here is according to the manufacturer's instructions for the TNT-coupled rabbit reticulocyte lysate system (Promega) and previous publications (14). We and others have used preSSU as the model substrate for binding and import experiments.

⁵The typical yield of chloroplasts from two plates of 8–10 g tissue is 200–400 µg chlorophyll.

1. Rapidly thaw all transcription-translation reagents and place on ice immediately before performing the reaction.
2. Place a 1.5-mL microcentrifuge tube on ice and add the following reagents in the following order: 25 μ L TNT rabbit reticulocyte lysate, 2 μ L TNT reaction buffer, 4 μ L [35 S] methionine (1,000 Ci/mmol at 10 mCi/mL), 1 μ L T7 TNT RNA polymerase, 1 μ L amino acid mix minus methionine, and 1 μ L RNase inhibitor.
3. Add 1 μ g of pET21a-preSSU plasmid (4). The volume of plasmid should be kept to a minimum (1 μ L). (For isolation of plasmid, see Note 6.)
4. Add nuclease-free water to bring the final volume to 50 μ L.
5. Incubate the reaction at 30°C for 90 min without shaking.
6. After the translation reaction, the translation mixture is diluted with 450 μ L of ice-cold HS and gel-filtered using Sephadex G-25 to remove free nucleotides. (For the method for gel filtration, see Note 7.) Alternatively, free nucleotides can be removed free by dialysis (24) (see Note 8).

3.4. Generation of Protein Import Intermediates and Chemical Cross-Linking to Map Translocon–Preprotein Interactions

To examine the components involved in preprotein binding and the formation of early import intermediates, we have employed a chemical cross-linking approach using the homobifunctional cysteine cross-linker, DTME (13). preSSU contains five cysteines at positions 55, 96, 132, 145, and 167. After the cross-linking reaction, detergent-solubilized chloroplasts are immunoprecipitated with antibodies to TOC/TIC components to identify components that interact with the preprotein at each stage. The results of a typical cross-linking reaction in which preSSU is bound to chloroplasts in the presence of GTP is shown in Fig. 1. Immunoprecipitation of the samples with anti-Toc159 or anti-Toc33 antibodies under denaturing conditions demonstrates that the method can distinguish interactions between preSSU and the two TOC receptors under these conditions.

3.4.1. Generation of Protein Import Intermediates

1. Energy-independent binding: Dilute isolated, energy-depleted chloroplasts corresponding to 200 μ g of chlorophyll (see Subheading 3.2, step 2) with HS buffer and 0.1 volume 10 $\times$ MMX (50 μ L) to a final volume of 500 μ L. Initiate the binding reaction by adding 10–50 μ L of energy-depleted in vitro translation products

⁶To isolate plasmids for coupled transcription-translation, we typically use a commercially available plasmid isolation kit (Qiagen Plasmid Midi kit). This system invariably results in high-quality plasmid lacking contaminants that can interfere with the transcription or translation reactions.

⁷A volume of 1 mL of Sephadex G-25 reconstituted in water is pipetted into the barrel of a 1-cc syringe (Becton, Dickinson & Company). The tip is plugged with glass wool to allow solutions but not the Sephadex from passing through the syringe. The syringe is inserted into a 15-mL conical centrifuge tube and the Sephadex is washed by the application of five volumes of HS buffer. The column is centrifuged for 1 min at 1,000 $\times$ g in a tabletop clinical centrifuge between each application of buffer to force out the excess liquid. A microcentrifuge tube with cap removed is placed at the bottom of the 15-mL tube and the syringe inserted so that the tip is inserted into the microfuge tube. The translation mixture is layered onto the Sephadex and the syringe is centrifuged for 1 min at 1,000 $\times$ g at 4°C. The filtered translation mix is recovered in the microfuge tube and can be stored at –80°C until use for at least 2 weeks.

⁸As an alternative method to gel filtration for the removal of free nucleotides, diluted translation mixtures can be dialyzed in 10,000 molecular weight cut-off dialysis tubing (SpectroPor 1 or 2) and incubated with 100 volumes of HS buffer at 4°C. The buffer should be changed two times with fresh HS buffer every 1 h and the dialysis should not exceed 4 h.

(equivalent to at least 25,000 cpm) in HS buffer and incubate at 26°C for 5 min in the dark (14).

2. GTP-stimulated binding: Dilute ATP-depleted chloroplasts corresponding to 200 µg of chlorophyll (see Subheading 3.2, step 1) with HS buffer and 0.1 volume 10× MMX (50 µL) to a final volume of 500 µL. Add GTP to the chloroplast suspension at a final concentration of 0.1 mM and incubate the mixture at 26°C for 5 min in the dark. Initiate the binding reaction by adding 10–50 µL of energy-depleted in vitro translation products (equivalent to at least 25,000 cpm) in HS buffer and incubate at 26°C for 5 min in the dark (14).
3. Early import intermediate: Dilute ATP-depleted chloroplasts corresponding to 200 µg of chlorophyll (see Subheading 3.2, step 3) with HS buffer and 0.1 volume 10× MMX (50 µL) to a final volume of 500 µL. Add GTP to the chloroplast suspension to a final concentration of 0.1 mM and ATP to a final concentration of 0.1 mM. Incubate the mixture at 26°C for 5 min in the dark. Initiate the reaction by adding 10–50 µL of energy-depleted in vitro translation products (equivalent to at least 25,000 cpm) in HS buffer and incubate at 26°C for 5 min in the dark.

3.4.2. Chemical Cross-linking to Map Translocon–Preprotein Intermediates

1. Initiate cross-linking in energy-independent and GTP-dependent binding reactions (see Subheading 3.4.1) with the addition of 2.5 mM DTME.
2. For the early import intermediate (see Subheading 3.4.1), the chloroplasts are isolated through a 35% Percoll cushion to remove excess translation product prior to initiating cross-linking. Layer the reaction over 1 mL of 35% Percoll cushion in HS buffer in a microcentrifuge tube. Centrifuge the sample for 1 min on low speed in the microcentrifuge. Wash the chloroplast pellet twice with 1 mL of HS buffer. Resuspend the chloroplasts in 475 µL of HS buffer and add 25 µL of DTME to a final concentration of 2.5 mM.
3. Incubate the cross-linking reactions on ice in the dark for 45 min.
4. Dilute the reactions tenfold with ice-cold HS buffer, collect the chloroplasts by centrifugation for 1 min at high speed in a microcentrifuge, and wash once with HS buffer before immunoprecipitation.

3.4.3. Immunoprecipitation of Cross-Linked Import Intermediates

1. For immunoprecipitation under denaturing conditions, dissolve chloroplasts (150 µg chlorophyll) (see Subheading 3.4.2) in 80 µL of 2% SDS containing 2% PIC by vigorous mixing.
2. Heat the samples for 5 min at 97°C and cool to room temperature.
3. Dilute the samples with 720 µL of dilution buffer and incubate on ice for 5 min.
4. After a 30-min centrifugation at $18,000 \times g$ at 4°C, divide the supernatant into two equal volumes and incubate with 2 µg/mL affinity-purified Toc159 A-domain antibody or Toc33 antibody for 16 h at 4°C.

5. Add 20 μL of packed protein A–Sepharose resin in dilution buffer to each sample and incubate for 2 h at 4°C with constant rotation.
6. Wash the Sepharose five times with washing buffer A and analyze by SDS-PAGE.
7. The amounts of precipitated receptors and bound preSSU are determined by immunoblotting and phosphorimaging, respectively (Fig. 1).

3.5. Chemical Cross-Linking to Map Translocon Assembly and Dynamics

To examine the translocon assembly and dynamics, we have employed a chemical cross-linking approach using the homobifunctional amine cross-linker, DSP (4, 5, 25). Although the interactions of several of the core TOC–TIC components (Toc75, Toc159, Toc33, and Tic110) appear to be quite stable, the association of these proteins with other components of the import apparatus (e.g., the chaperone machinery) is dynamic. As a result, detection of these interactions requires stabilization with covalent cross-linkers. After the cross-linking reaction, detergent-solubilized chloroplasts are immunoprecipitated with antibodies to atTic110 to identify components that interact with atTic110. An example of the stabilization of the Tic40–Hsp93 motor complex with Tic110 using DSP cross-linking is shown in Fig. 2. DSP has the advantage of containing a reducible disulfide that allows cleavage of the cross-links for resolution of individual polypeptides after immunoprecipitation.

1. For cross-linking, isolated chloroplasts (see Subheading 3.1) are incubated in the dark for 30 min at 26°C to deplete internal ATP and reducing potential. If energy-dependent interactions are of interest, the energy state of the chloroplasts can be adjusted accordingly by the exogenous addition of nucleoside triphosphates (NTPs) (see Subheading 3.4.1).
2. Resuspend chloroplasts corresponding to 200 μg of chlorophyll in 475 μL of HS buffer and add 25 μL of DSP to a final concentration of 0.25–1 mM on ice for 15 min in the dark. For the stabilization of Tic40 and Hsp93 with the translocons, we use a maximum of 1 mM DSP.
3. Quench the reaction by incubating with glycine at a final concentration of 50 mM on ice for an additional 15 min.
4. Collect the chloroplasts by centrifugation for 1 min at high speed in a microcentrifuge and wash the pellet once with HS buffer before fractionation.
5. Lyse the chloroplasts by suspension in HS buffer to a concentration of 0.5–1 mg chlorophyll/mL and dilute with five volumes of 2 mM EDTA to induce osmotic lysis. Mix the lysate vigorously and incubate on ice for 10 min.
6. Collect the membrane fraction by centrifugation at $18,000 \times g$ for 30 min at 4°C in a microcentrifuge.
7. For immunoprecipitation, dissolve the total membranes (200 μg of chlorophyll) from DSP cross-linked chloroplasts in 200 μL TES buffer for 10 min on ice.
8. After a 30-min centrifugation at $18,000 \times g$ at 4°C in a microcentrifuge, incubate the supernatant with 2 $\mu\text{g}/\text{mL}$ affinity-purified Tic110 antibody for 16 h at 4°C.

9. Add 20 μ L of packed protein A–Sepharose resin in TES buffer to each sample and incubate for 2 h at 4°C with constant rotation.
10. Wash the Sepharose five times with washing buffer B and elute the bound proteins directly with 50 μ L of SDS-PAGE buffer.
11. Analyze the immunoprecipitates by SDS-PAGE and immunoblot with the antibodies to TOC/TIC components shown in Fig. 2.

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Fig. 1.

GTP-dependent binding of preSSU to components of the TOC translocons. Energy-depleted, in vitro-translated [³⁵S]preSSU was incubated with isolated chloroplasts in the presence of 0.1 mM GTP to promote GTP-dependent binding. The homobifunctional cross-linker DTME was added to cross-link the bound preprotein to TOC components. The chloroplasts were detergent solubilized under denaturing conditions and proteins immunoprecipitated with Toc159 or Toc33 affinity-purified antibodies. The immunoprecipitates were analyzed by SDS-PAGE and phosphorimaging to quantify co-immunoprecipitated [³⁵S]preSSU or by immunoblotting to detect Toc159 or Toc33.

Fig. 2.

Stabilization of the association of Hsp93 and Tic40 with TOC–TIC complexes. Detergent extracts from isolated chloroplasts that had been treated with DSP at the concentrations indicated (lanes 2–4) or DMSO as a control (lane 1) were subjected to immunoprecipitation with anti-Tic110 antibodies. The immunoprecipitates were immunoblotted with the antibodies indicated at the right of the figure.