DEVELOPMENT OF A PRECURSOR IN A SOLUBLE FORM FOR PROTEIN IMPORT INTO CHLOROPLASTS

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ABSTRACT

Most of chloroplastic proteins are encoded in the nuclear genome. After majority of them are synthesized as precursors in the cytosol, precursors translocate through the outer and inner envelope membranes via translocation machinery (translocon) embedded in these membranes. Our current knowledge of translocon is mainly gained from the early stages of translocation by analysing the protein translocation intermediates (PTIs) formed in vitro under limited energy conditions. Once precursor proteins are released from the PTIs in the presence of a high level of ATP, it is impossible to suspend the movement of precursors until translocation is completed. Thus little is known regarding molecular mechanisms at the latter stages of translocation. However, if precursors are fused with the tightly folded domain to introduce steric hindrance, these precursors may plug the translocon channel under a translocation condition, as a result, PTIs at the latter stage of translocation will be formed. In order to isolate PTIs enough for biochemical analysis, a large quantity of precursors in a soluble form are required. Therefore, we made an attempt to obtain the soluble precursor protein with tightly folded domain by overexpressing in Escherichia coli. Because dihydrofolate reductase (DHFR) is tightly folded in the presence of methotrexate (MTX), we designed and prepared the plasmid carrying the gene for the artificial precursor protein consisted with chloroplastic targeting sequence and various tags along with DHFR from E. coli. After overexpression in E. coli cells, this precursor was recovered in a soluble fraction. When this precursor was applied for in vitro chloroplastic protein import, the precursor was imported into chloroplasts.

KEYWORDS: Protein Import Into chloroplast, Precursor Protein, Steric Hindrance

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INTRODUCTION

Most of nuclear encoded chloroplastic proteins are synthesized as precursors with chloroplastic targeting sequences, transit peptides, at their amino-termini. Majority of them are imported into chloroplasts through the outer and the inner envelope membrane through translocons embedded in these membranes (for reviews, see Hormann et al., 2007; Inaba and Schnell, 2008; Jarvis, 2008; Richardson et al., 2014). Based on energy requirement, the process of protein import into chloroplast can be separated into at least two steps, ‘docking’ and ‘translocation’ (Olsen et al., 1989; Olsen and Keegstra, 1992). Docking involves irreversible binding of precursor to the chloroplastic surface and form protein translocation intermediates (PTIs) in which precursors are trapped in translocon under stringent energy conditions, i.e. low concentrations of GTP and/or ATP, and a low temperature. On the other hand, in the presence of a high concentration of ATP (>1 mM) at higher temperature, precursors translocate through both envelope membranes. During translocation or after translocation is completed, their
transit peptides are cleaved by the stromal processing peptidase (Chua and Schmidt, 1978; Oblong and Lamppa, 1992). From our previous works, docking step is shown to be further separated into three stages (I, II, and III) in which the extent of precursor translocation differs depending on the different energy conditions: in the presence of GTP alone (Stage I); in the presence of ATP at 4°C (Stage II); in the presence of a low level of ATP at higher temperature (Stage III) (Inoue and Akita, 2008ab; Akita and Inoue, 2009). Furthermore, the entire length of the transit peptide was found to be inserted into the outer envelope membrane at the Stage II and III (Inoue and Akita, 2008ab; Akita and Inoue, 2009).

During protein import process, precursor proteins are expected to interact sequentially with the translocon components. Such molecular interactions can be easily analysed if PTIs are formed. Therefore, many knowledges regarding translocon have been gained through the analysis of the PTIs at the docking step (Akita et al., 1997; Hirsch et al., 1994; Kessler et al., 1994; Kouranov and Schnell, 1997; Ma et al., 1996; Nielsen et al., 1997; Perry and Keegstra, 1994; Schnell et al., 1994). However, little is known about the latter stages of translocation. Once after precursors are launched from the PTIs formed during the docking step, in the presence of a high level of ATP at a higher temperature, precursors keep passing through the translocon channel without an interruption, which make the analysis of interactions between precursors and the translocon components difficult. However, if precursors with steric hindrance can plug the translocon channel during translocation to form the PTIs artificially, understandings about the whole process of protein import into chloroplasts are expected to be advanced by analysing such PTIs. Preparation of such precursors will be achieved if a precursor is fused to a tightly folded domain. In addition, certain amount of precursor protein is required for the analysis of PTIs biochemically. We have developed the in vitro chloroplastic protein import system which can handle a large quantity of precursors (Akita and Inoue, 2009; Inoue et al., 2008; Sattasuk et al., 2011). In this system, precursors used were urea-solubilized recombinant proteins overexpressed in E. coli cells, recovered as inclusion bodies. Since the precursor with steric hindrance is to fuse a tightly folded domain, the precursor must be in a soluble form when expressed in E. coli cells. In order to obtain such precursor protein, we designed the artificial precursor consisted with a transit peptide, various tags, and a soluble protein which can fold tightly. The designed precursor was successfully expressed as a soluble protein in E. coli cells.

**METHODS**

**Construction of the Precursor Expression Plasmid**

The expression plasmid for the precursor (TP-HA-mSS-H6-B, Figure 1A) used for a control was modified from the expression plasmid coding prSSC0HAHAH (Inoue et al., 2008), in which all three cysteine residues in the precursor form of small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (prSS) from pea (Pisum sativum) was replaced with serine residues and the tandem tag of the double human influenza hemagglutinin protein (HA) epitope tag and the hexahistidine (H6) tag, by gene manipulation as follows. Firstly, the biotin acceptor peptide (BAP) was fused to the carboxy terminal of prSSC0HAHAH, followed by the replacement of double HA tag with TEV protease recognition site (TEV) and the epitope tag derived from vesicular stomatitis virus glycoprotein (VSV-G). Finally, the HA epitope tag was inserted in the fourth and fifth amino acid residue of mature part of prSS. Furthermore, for the efficient in vivo biotinylation at BAP, the gene for biotin ligase (BirA) from E. coli with ribosome binding site was inserted at the 3’ of the stop codon of the gene for TP-HA-mSS-H6-B on the expression plasmid to form an artificial operon. The expression plasmid for the soluble precursor protein (TP-HA-r1-DHFR-HAT-B, Figure 1B) was prepared from this plasmid as follows. Firstly, the histidine affinity tag (HAT) (Chaga et al., 1999) was substituted for H6 tag. Then mature part of SS from fifth
amino acid residue (mSS) was replaced with dihydrofolate reductase (DHFR) from *E. coli*. Finally, one unit of random coil linker (r1; five repetition of penta-peptide of GGGGS) was inserted between the HA epitope tag and DHFR.

### Protein Expression

To overexpress both the control and the soluble precursor proteins, 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to the culture of *E. coli* BL21(DE3) cells carrying the respective expression plasmid in LB broth containing 50 μg mL⁻¹ carbenicillin when it reached the mid-logarithmic growth phase, followed by a further incubation for 1 h at 37°C. Harvested *E. coli* cells were resuspended in a buffer (50 mM Tris-HCl, pH 7.5, 10 % glycerol) and after the cells were ruptured by sonication, the soluble and the insoluble fractions were separated by centrifugation at 20,000 × g for 10 min at 4°C. The insoluble fraction was washed three times with H₂O. In case for the control precursor, it was recovered in the inclusion bodies, so that the final precipitate was solubilized with S-buffer (8 M Urea, 25 mM HEPES-KOH, pH 7.5, 50 mM KCl, 2 mM MgCl₂). For the soluble precursor, the soluble fraction was applied for purification as described below.

### Protein Purification

The soluble precursor protein was purified from the cell lysate by 100 μL of Ni-NTA agarose (Qiagen). Cell lysate (200 μL) was diluted twice with binding buffer (50 mM NaH₂PO₄, pH 7.8, 300 mM NaCl) and mixed with Ni-NTA resins which were pre-equilibrated with binding buffer. After 1 h with shaking, the mixture was loaded into a column and flow through was collected by gravity flow. Column was washed twice with 1 mL of wash buffer (binding buffer with 10 mM imidazole) and then precursor protein was eluted with the 500 μL of binding buffer containing 150 mM imidazole. Buffer exchange to import buffer (50 mM HEPES-KOH, pH 8.0, 330 mM sorbitol) was carried out by spin column packed (1 mL bed volume) with Sephadex G-25 (GE Healthcare).

### Chloroplast Isolation and Import Assay

Chloroplasts were isolated from pea seedlings as described previously (Bruce et al., 1994) and suspended in import buffer to yield a suspension containing 1 mg of chlorophyll mL⁻¹. When chloroplasts were subjected to the import assay, 20 μL of the chloroplast suspension was pre-incubated with 2.5 μM nigericin in the dark for 10 min at 25°C, followed by 4-fold dilution with import buffer with 1.25 mg mL⁻¹ BSA, 31.25 mM DTT, 6.25 mM MgCl₂, and 3.125 mM ATP and incubated in the dark for 5 min at 25°C. Eighteen μL of import buffer and 2 μL of 10 μM control precursor protein solubilized in S-buffer or 20 μL of the buffer exchanged purified soluble precursor solution was then added to initiate import reaction, followed by the incubation in the dark for 20 min at 25°C. The reaction mixture was loaded onto 40 % Percoll in import buffer and centrifuged (1500 × g) at 4°C for 5 min to recover intact chloroplasts, which were washed once with import buffer. Chloroplasts were suspended in sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE) or subjected to fractionation as described below.

### Fractionation of Chloroplasts

After recovery from the 40 % Percoll cushion and the subsequent wash as described above, chloroplasts were suspended in HM buffer (25 mM HEPES-KOH, pH 8.0, 2 mM MgCl₂) to contain 1 mg of chlorophyll mL⁻¹ to lyse hypotonically, and then they were incubated on ice for 10 min in the dark. Chloroplast suspension was adjusted to a final concentration of HM-0.2 with HM-2 (HM buffer with 2 M sucrose) and fractionated into the soluble and the membrane fractions as previously described (Perry et al., 1991).
Thermolysin Treatment

After incubation for 20 min at 25 °C under import conditions, chloroplasts were recovered by centrifugation and resuspended in the import buffer containing 1 mM CaCl₂. Half fraction of this chloroplast suspension was incubated with thermolysin with a final concentration of 200 µg mL⁻¹ for 30 min on ice. The reaction was stopped by addition of EDTA to a final concentration of 10 mM. Intact chloroplasts from the thermolysin treated and untreated fractions were obtained by centrifugation through 40 % Percoll containing 10 mM EDTA, washed once with import buffer containing 10 mM EDTA and finally suspended in SDS-PAGE sample buffer which contains 10 mM EDTA and boiled immediately.

Electrophoresis and Immunoblotting

Protein samples were analysed by SDS-PAGE (Laemmli, 1970) after mixed with sample buffer, followed by boiling for 5 min. Once electrophoresis was completed, gels were stained with Coomassie Brilliant Blue (CBB) R-250 or subjected to immunoblotting. Immunoblotting with anti-HA monoclonal antibody (M180 from MBL) as a primary antibody was performed as described previously (Inoue et al., 2008).

RESULTS AND DISCUSSION

Designing the Soluble Precursor Protein

Entire view of protein import process into chloroplasts has not been grasped yet. The study on protein import into chloroplasts have been mainly focused on the analysis of the early stages of protein translocation through the analysis of protein translocation intermediates (PTIs) which are formed in vitro by precursor proteins which irreversibly bind to the protein import machinery (translocon) under stringent energy conditions. Once precursors are released from PTIs, it is impossible to capture import substrates until translocation is over. Therefore, if we can isolate PTIs at the latter stages of import, knowledge regarding this subject will definitely extend. To this end, we made an attempt to prepare precursor proteins with tightly folded domain whether the respective domain could plug translocon channel to form PTIs.

We have developed the in vitro chloroplastic import system which could accommodate the large scale reaction by the use of recombinant precursors overexpressed in E. coli cells (Akita and Inoue, 2009; Inoue et al., 2008; Sattasuk et al., 2011). Because all of E. coli overexpressed precursors prepared so far, including the fusion protein of GFP attached with the transit peptide of prSS, were recovered in the inclusion bodies and had to be solubilized in 8 M urea prior to the reaction (Akita and Inoue, 2009; Inoue and Akita, 2008ab; Inoue et al., 2008; Ratnayake et al., 2008ab; Sattasuk et al., 2011). However, denaturation step should be avoided, if the precursor with a tightly folded domain was applied for plugging translocation channel. Therefore, we thought out how we could get the precursor protein in a soluble form and reached to the conclusion that the precursor protein would be assembled with soluble components. Since DHFR is tightly folded in the presence of methotrexate (MTX) (Rajagopalan et al., 2002), in addition, to avoid the use of a gene from heterologous origin, DHFR from E. coli was selected to be a major component of the mature part of the precursor. For a detection and purification purpose, HA epitope tag, BAP, and HAT were chosen. HAT, instead of H6 tag, was picked because it was considered to be more soluble than artificial H6 tag (Chaga et al., 1999). These components were replaced with parts of the mature form of the precursor TP-HA-mSS-H6-B (Figure 1A) carrying the transit peptide of pea prSS, which were recovered as an insoluble form when expressed in the E. coli cells. In addition, to extend the distance between the transit peptide and DHFR, one unit of the random coil linker (r1) consisted with the quintuple penta-peptides of GGGGS ((G₄S)₅) was inserted to complete the precursor construction of TP-HA-r1-DHFR-HAT-B (Figure 1 B).
Protein Expression

TP-HA-r1-DHFR-HAT-B was overexpressed by adding IPTG during a mid-logarithmic phase (Figure 2, lane 1) and incubated for 1 h at 37°C (Figure 2, lane 2). Bacterial culture was not incubated for more than 1 h after IPTG induction to avoid degradation of the expressed protein in a soluble form by endogenous proteases of E. coli. Harvested E. coli cells were fractionated into the soluble (Figure 2, lane 3) and the insoluble (Figure 2, lane 4) fractions after ruptured by sonication. Most of overexpressed TP-HA-r1-DHFR-HAT-B was observed in the soluble fraction (Figure 2, lane 3), indicating that we could obtain the precursor in a soluble form. Immunoblotting by anti-HA monoclonal antibody and streptavidin, TP-HA-r1-DHFR-HAT-B was detected at the same size as observed in CBB stained gel (data not shown). These results indicated that TP-HA-r1-DHFR-HAT-B was in vivo biotinylated at BAP in E. coli cells.

Soluble Precursor Purification

Now the precursor was recovered in the E. coli lysate, the precursor was purified under native conditions with Ni-NTA resins, by utilizing HAT tag present in the precursor. TP-HA-r1-DHFR-HAT-B was bound to the resin and eluted (Figure 3, lane 4). An additional prominent protein along with few more proteins have been found to be co-purified in addition to the precursor protein. Co-purification of this prominent protein was might be due to the presence of an additional methionine just after the cleavage site in the precursor, which might be acting as an additional translation initiation site. Other minor co-purified proteins were seems to be the N-terminal truncations of the precursor protein as they have been purified with the HAT tag which was situated towards the C-terminus end of the precursor.

In vitro Chloroplastic Protein Import assay

In order to determine whether engineered precursor protein was targeted to chloroplasts, we employed the in vitro chloroplastic protein import assay, utilizing the purified TP-HA-r1-DHFR-HAT-B (Figure 4A, lanes 4 and 5) and urea-solubilized TP-HA-mSS-H6-B (Figure 4A, lanes 2 and 3) as a control. In addition the mock import assay was performed (Figure 4A, lane 1). After the import reaction, smaller-sized processed band was produced from both precursors (Figure 4A, lanes 3 and 5). These results suggested that TP-HA-r1-DHFR-HAT-B was translocated enough for its cleavage site was reached to the stromal space where stromal processing peptidase cleaved the transit peptide to produce the mature form. However, from these results, we were unable to conclude that whether TP-HA-r1-DHFR-HAT-B was fully translocated into the stromal space.

Therefore, we made an attempt to analyse the nature of the processed protein by fractionating chloroplasts into the soluble (Figure 4B, lanes 1, 3, and 5) and the membrane (Figure 4B, lanes 2, 4, and 6) fractions after chloroplasts were lysed. The processed band was observed in both fractions in case for TP-HA-mSS-H6-B (Figure 4B, lanes 3 and 4). Although Rubisco is the soluble protein, substantial amount of endogenous Rubisco was usually recovered in the membrane fraction (data not shown). Since TP-HA-mSS-H6-B was modified from prSS, recovery of mature protein produced from TP-HA-mSS-H6-B in membrane fraction was highly possible. In the case of TP-HA-r1-DHFR-HAT-B, the processed band was recovered in the soluble fraction (Figure 4B, lane 5). In addition, processed proteins from both the precursors were found to be resistant to thermolysin (data not shown). These results clearly indicated that these processed proteins were completely translocated into the chloroplast.
CONCLUSIONS

The engineered precursor protein carrying DHFR in its mature part in the present study was obtained successfully in the soluble form and found to be import competent. Further, with this tightly folding DHFR domain, this precursor can be considered as an important tool for plugging the translocon channel. If this precursor could form the PTIs during the translocation stage in presence of MTX then the carboxy terminal biotinylated BAP will become the powerful tool for fishing the PTIs after solubilisation of chloroplastic membranes by a detergent.

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APPENDICES

A. TP-HA-mSS-H6-BAP

| TP(prSS) | HA | mSS | TEV | VSV-G | H6 | BAP |

B. TP-HA-r1-DHFR-HAT-BAP

| TP(prSS) | HA | r-coil | DHFR | HAT | BAP |

Figure 1: Diagrams of the Precursor used in this Study

A, TP-HA-mSS-H6-B. This precursor was modified from prSSC0HAHAH (Inoue et al., 2008) to possess the following components; HA epitope tag was inserted into fourth and fifth amino acid residue of mature SS, TEV protease recognition site (TEV), the VSV-G epitope tag, hexa histidine (H6) tag, and biotin acceptor peptide (BAP). This precursor was used as a control precursor for TP-HA-r1-DHFR-HAT-B.

B, TP-HA-r1-DHFR-HAT-B. This precursor was prepared from TP-HA-mSS-H6-B as follows; mSS-H6 part was replaced with random coil linker (r-coil; 5 repeats of GGGGS), DHFR from E. coli and histidine affinity tag (HAT).

Figure 2: Overexpression of TP-HA-r1-DHFR-HAT-B

Overexpression of TP-HA-r1-DHFR-HAT-B is described in “Methods” section. Whole proteins from E. coli cells just before adding IPTG (lane 1) and 1 h of incubation after adding IPTG (lane 2) were solubilized and denatured with the sample buffer of Laemmli’s system (Laemmli, 1970) and analyzed by SDS-PAGE, followed by the CBB staining. The supernatant after sonication (lane 3) and the precipitates after final wash (lane 4) were also analysed by SDS-PAGE, followed by the CBB staining. Molecular mass (kDa) is shown on the left and the position of TP-HA-r1-DHFR-HAT-B is depicted as “p”.

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The *E. coli* lysate (lane 1) was mixed with Ni-NTA agarose and incubated for 1 h with shaking. The mixture was poured into the column and flow through (lane 2) was recovered. After washing resin (lane 3), bound protein was eluted by the buffer containing 150 mM imidazole (lane 4). Each fraction was analyzed by SDS-PAGE, followed by CBB staining. Molecular mass (kDa) is shown on the left and the position of TP-HA-r1-DHFR-HAT-B is depicted as “p”.

![Figure 3: Purification of TP-HA-r1-DHFR-HAT-B by Ni-NTA Agarose](image)

A. Import buffer (-Precursor), TP-HA-mSS-H6-B, and TP-HA-r1-DHFR-HAT-B were mixed with energized chloroplast suspension. After 20 min incubation at 25°C, intact chloroplasts were recovered through 40% Percoll and then washed once. Chloroplasts were lysed with sample buffer of Laemmli’s system (Laemmli, 1970), followed by boiling for 5 min. Samples were separated by SDS-PAGE and analysed by immunoblotting. The blots were decorated with the monoclonal antibody against HA epitope tag. Molecular mass (kDa) is shown on the left and the position of TP-HA-mSS-H6-B and TP-HA-r1-DHFR-HAT-B is depicted as “p”, while processed band is depicted as “m”. 10% of the
precursor applied for the import reaction (lanes 2 and 4) was loaded onto the gel.

B. After import reaction was performed with TP-HA-mSS-H6-B, TP-HA-r1-DHFR-HAT-B, and buffer only sample (-Precursor), chloroplasts were lysed hypotonically and fractionated into the soluble (lanes 1, 3, and 5) and the membrane fractions (lanes 2, 4, and 6). Each fraction was separated with SDS-PAGE (Laemmli, 1970) after adding sample buffer and analysed by immunoblotting. The blots were decorated with the monoclonal antibody against HA epitope tag. Molecular mass (kDa) is shown on the left and the position of TP-HA-mSS-H6-B and TP-HA-r1-DHFR-HAT-B is depicted as “p”, while processed band is depicted as “m”.