**Sec-dependent Pathway and ΔpH-dependent Pathway Do Not Share a Common Translocation Pore in Thylakoidal Protein Transport**

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Tomomi Asai‡, Yoshihiro Shinoda‡, Tetsuya Nohara‡, Tohru Yoshiiha‡,¶ and Toshiya Endo‡

*From the ‡Department of Chemistry, Faculty of Science and the ¶Research Center for Materials Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan

Thylakoidal proteins of plant chloroplasts are translocated to thylakoids via several different pathways, including the ΔpH-dependent and the Sec-dependent pathways. In this study, we asked if these two pathways utilize a common translocation pore. A fusion protein consisting of a 23-kDa subunit of the oxygen evolving complex and *Escherichia coli* biotin carboxyl carrier protein was biotinylated in *E. coli* cells and purified. When incubated with isolated pea thylakoids in the absence of avidin, the purified fusion protein was imported into the thylakoids via the ΔpH-dependent pathway. However, in the presence of avidin, the fusion protein became lodged in the thylakoid membranes, with its N terminus reaching the thylakoidal lumen, while its C-terminal segment complexed with avidin exposed on the thylakoidal surface. The translocation intermediate of the fusion protein inhibited the import of authentic 23-kDa subunit, suggesting that it occupies a putative translocation pore for the ΔpH-dependent pathway. However, the intermediate did not block import of the 33-kDa subunit of the oxygen evolving complex, which is a substrate for the Sec-dependent pathway. These results provide evidence against the possibility of a common translocation pore shared by the Sec-dependent pathway and the ΔpH-dependent pathway.

Nuclear-encoded thylakoidal proteins are synthesized in the cytosol, are imported into the chloroplast stroma, and are subsequently inserted into or translocated across the thylakoid membranes. Recent evidence has shown that thylakoidal proteins are transported to the thylakoids via several different pathways, including the Sec-dependent and the ΔpH-dependent pathways (1, 2). In bacterial cells, secretory proteins are translocated across the cytoplasmic membrane through a channel consisting of SecY/E/G proteins with the aid of a translocation ATPase, SecA. In chloroplasts, homologues of SecA (cpSecA), SecY (cpSecY) and SecE (cpSecE) have been identified and shown to mediate transport of a class of thylakoid luminal proteins *in vitro* and *in vivo* (3–12). Another class of thylakoid luminal proteins is translocated across the thylakoid membranes via a transport pathway that requires ΔpH across the thylakoid membranes as a sole energy source and Hef106 protein in the thylakoid membranes (13–15). This ΔpH pathway appears to have ability to translocate folded proteins that are not accepted by the Sec-dependent pathway (16–18). Recent studies have revealed that a protein transport pathway similar to the thylakoidal ΔpH-dependent pathway is present in bacterial cells (19–21).

Although the Sec-dependent and the ΔpH-dependent pathways appear to utilize some pathway-specific components, they could share common translocation pore in the thylakoid membranes. *In vitro* competition experiments have shown that the import of a certain substrate protein into isolated thylakoids is effectively competed with saturating amounts of another competitor protein which utilizes the same thylakoidal transport pathway as the substrate protein but not with competitor proteins using other pathways (21). However the competition could occur only in the rate-limiting step of the transport, and the passage through a possible common translocation pore might not correspond to this step. A maize mutant lacking cpSecY shows more severe defects in thylakoid biogenesis than mutants lacking cpSecA, suggesting a possibility that cpSecY is involved in the thylakoidal protein transport along the pathways other than the Sec-dependent one (12).

One of the crucial tests for the possibility of the common translocation pore shared by different thylakoidal transport pathways would be to generate a saturating amount of a translocation intermediate that occupies most of the translocation pores for one pathway and to see its competition effects on the protein transport along other pathways. Such a translocation intermediate will span the thylakoid membranes with topology in which its N terminus reaches the thylakoid lumen while its C terminus remains outside the thylakoids. Since precursor proteins bearing avidin, which forms a stable tetrameric structure and tightly binds biotin, have been successfully used to generate a translocation intermediate spanning the mitochondrial membranes (23) and that spanning the chloroplast envelope (24), we supposed that the bulky avidin would not be accommodated by the translocation pore for the ΔpH pathway. In this study, a fusion protein (23K-BCCP) consisting of a wheat 23-kDa protein of the oxygen evolving complex (23K) and *Escherichia coli* biotin carboxyl carrier protein (BCCP), which is efficiently biotinylated *in vivo* (25), was expressed in *E. coli* cells. *In vitro* import experiments with isolated pea thylakoids in the presence of avidin, the purified fusion protein became lodged in the thylakoid membranes, with its N terminus reaching the lumen and its C-terminal segment complexed with avidin exposed on the thylakoid surface. The translocation

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‡ Research Fellow of the Japan Society for the Promotion of Science.

¶ To whom correspondence should be addressed: Research Center for Materials Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan. Tel.: 81-52-789-2950; Fax: 81-52-789-2947; E-mail: tyoshihi@biochem.chem.nagoya-u.ac.jp.

The abbreviations used are: 23K and 33K, 23- and 33-kDa subunits of oxygen evolving complex, respectively; BCCP, biotin carboxyl carrier protein; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
intermediate of the fusion protein specifically inhibited the translocation via the ΔpH pathway but not via the Sec pathway. These results provide strong evidence against the possibility of a common translocation pore shared by the two pathways.

EXPERIMENTAL PROCEDURES

Materials—Most of the reagents were purchased from Nacalai Tesque, Sigma, Takara, and Toyobo. Chloroplasts, chloroplast lysate prepared by osmotic lysis, thylakoids, and stromal fraction were prepared from pea seedlings (Pisum sativum, var. Alaska) as described previously (3).

Plasmids—A DNA fragment for 69–156 amino acid residues of BCCP (25) was amplified from E. coli chromosome and 3’-terminally fused with that for a c-Myc epitope by PCR. The fragment was inserted into pET-21d (Novagen) at the HindIII/Xhol site to yield pET-BCCP/Mhd. A DNA fragment encoding the processing intermediate form of wheat 23K (i23K) (3) was amplified by PCR and inserted into the NcoI/EcoRI site of pET-BCCP/Mhd. The resulting plasmid, pET-i23K/BCCP, expresses an i23K-BCCP fusion protein shown in Fig. 1A. To construct pBirA, a biotin ligase overproduction plasmid, the birA gene with its own promoter was amplified by PCR from E. coli chromosome and was subcloned into pETV28 (Takara).

Production of Biotinylated i23K-BCCP—A BL21(DE3)pBirA strain harboring pET-i23K/BCCP was cultured in terrific broth with 0.1 mM isopropyl-β-D-thiogalactoside for 3 h. The cells were harvested and disrupted by sonication in 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 5 mM imidazole. A supernatant fraction from the centrifugation at 100,000 × g for 30 min was applied onto a His Bind Resin column (Novagen). i23K-BCCP was eluted from the column with a buffer containing 0.2 M imidazole, dialyzed against 20 mM HEPES-KOH, pH 8.0, and stored at −80 °C.

In Vitro Import into Pea Thylakoids—For import assays with radio-labeled substrates, [35S]-labeled processing intermediate forms of 23K (i23K) and 33K (i33K) were translated in a wheat germ cell-free system prepared from pea seedlings (Pisum sativum, var. Alaska) as described previously (3). The radiolabeled 23K and 33K were detected in the preparative SDS-PAGE, visualized by Coomassie Brilliant Blue. The biotinylated i23K-BCCP was detected by immunoblotting with anti-c-Myc monoclonal antibody 9E10.

RESULTS AND DISCUSSION

Preparation of a Biotinylated Substrate, i23K-BCCP—In order to halt the translocation of substrate proteins for the ΔpH-dependent pathway across thylakoid membranes, we utilized a biotinylated 23K protein complexed with a stably folded and bulky avidin tetramer. We introduced a biotin moiety into i23K, a wheat 23K protein with a truncated transit peptide lacking a chloroplast targeting signal, by expressing it as a fusion protein between i23K and residues 69–156 of BCCP in E. coli cells (Fig. 1A). It is known that Lys-122 in the above sequence is biotinylated in E. coli cells (25). A c-Myc/His<sub>6</sub>-tagged version of the fusion protein was expressed together with a BirA protein (biotin ligase) in E. coli BL21(DE3) cells, which were cultivated in the medium containing 0.1 mM biotin. The expressed 42-kDa fusion protein was soluble in the cytosol and was purified by metal affinity chromatography for the His<sub>6</sub> tag (Fig. 1B, lane 1). About 70% of the purified i23K-BCCP was found to be biotinylated (Fig. 1B, lane 2).

Next, we tested whether the purified fusion protein can be imported into isolated thylakoids via the ΔpH-dependent pathway like authentic i23K. Incubation of purified i23K-BCCP with isolated thylakoids converted it to a 39-kDa mature form (m23K-BCCP), which was protected against exogenous thermolysin (Fig. 1C, lanes 1 and 2), indicating that the fusion protein was imported into the thylakoids. The amount of protease-protected m23K-BCCP was not dependent on the extent of biotinylation of the BCCP segment (not shown). The transport into thylakoids was inhibited by nigericin (lanes 3 and 4), a protonophore dissipating ΔpH across the thylakoid mem-

![Fig. 1. Purification and characterization of in vitro biotinylated i23K-BCCP. A, i23K-BCCP fusion protein. The upper numbers indicate amino acid residue numbers for the fusion protein and the lower numbers those for the authentic precursor of 23K and authentic BCCP. B, two μg of purified i23K-BCCP was denatured in SDS-PAGE sample buffer, incubated without (lane 1) or with (lane 2) 10 μg of avidin at 25 °C for 5 min, and subjected to SDS-PAGE. Proteins were visualized by Coomasie Brilliant Blue. The biotinylated i23K-BCCP (●) migrates more slowly than the nonbiotinylated one (○) in lane 2, because the former forms a stable complex with avidin even in the presence of SDS. Lane 3 received avidin only. C, four μg of purified i23K-BCCP was incubated with 300 μg of chlorophyll of isolated pea thylakoids for 20 min at 25 °C. Reactions marked with nigericin (lanes 3 and 4) and NaN<sub>3</sub> (lanes 5 and 6) contained 0.5 μM nigericin and 10 mM NaN<sub>3</sub>, respectively. The reaction mixtures were divided into two, and the halves were treated with thermolysin (even-numbered lanes). 33K-BCCP was detected by immunoblotting with the anti-c-Myc antibody 9E10, intermediate form; m, mature form.
suggest that the mature form is associated with the thylakoid membranes through protein-protein interactions. This would be consistent with a possibility that the mature form spans the thylakoid membranes through a protein translocation pore.

**23K-BCCP-Avidin Complex Blocks the ΔpH-dependent Transport Pathway but Not the Sec-dependent Pathway**—The above results suggest that the i23K-BCCP complexed with avidin initiates translocation, but fails to complete it because the bulky avidin cannot be accommodated by the translocation pore for the ΔpH-dependent pathway. If the protease-sensitive mature form of 23K-BCCP represents a genuine translocation intermediate that is trapped in the translocation pore, it should block import of authentic substrates for the ΔpH pathway into the thylakoids. Thylakoids were thus incubated with large amounts of biotinylated i23K-BCCP in the presence or absence of avidin, recovered by centrifugation to remove excess amounts of the fusion protein and avidin, and subjected to the second incubation with radiolabeled i23K, an authentic substrate for the ΔpH dependent pathway. When i23K-BCCP was omitted during the first incubation, the thylakoids could import i23K efficiently whether or not avidin was present during the first incubation (Fig. 3A, lanes 2, 5, and 8). In contrast, when thylakoids were incubated with increasing amounts of 23K-BCCP in the presence of avidin, the reisolated thylakoids retained less ability to import i23K (Fig. 3A, lanes 3, 4, 6, and 7). The presence of avidin during the first incubation was essential to block the import of i23K during the second incubation (Fig. 3A, lanes 8–11). The first incubation with 10 μg of i23K-BCCP and avidin blocked the import of i23K in the second reaction to about 20% of the control reaction (Fig. 3A, lane 7). Therefore, we conclude that at least a part of protease-sensitive 23K-BCCP-avidin molecules represents a true translocation intermediate. The 23K-BCCP-avidin complex likely occupies most of the translocation pores for the ΔpH-dependent pathway.

Next, we asked if the translocation intermediate for the ΔpH-dependent pathway described above can block import of substrates for the Sec-dependent pathway. Thylakoids were treated as in Fig. 3A and subjected to the second incubation with radiolabeled i33K, an authentic substrate for the Sec-dependent pathway. The stromal fraction was added to the reaction to supply cpSecA, which is essential for the Sec-dependent transport (3, 4). When thylakoids were incubated with 10 μg of i23K-BCCP and avidin, which were sufficient to block 80% import of i23K during the second incubation, the reisolated thylakoids did not lose the ability to import i33K (Fig. 3B). Therefore, inhibition of thylakoidal protein transport by the arrested translocation intermediate of i23K-BCCP-avidin complex is specific for the ΔpH-dependent pathway. This means that the translocation pore for the ΔpH-dependent pathway is not shared by substrates for the Sec-dependent pathway. The ΔpH-dependent pathway and the Sec-dependent pathway most likely have their own translocation pores for protein passage across the thylakoid membranes.

Successful generation of the membrane-spanning translocation intermediate that occupies ΔpH-dependent translocation pores enables us to estimate the number of the import sites. Titration of the import sites for the ΔpH-dependent pathway in the thylakoid membranes with i23K-BCCP-avidin complex showed that the import sites were saturated by 73 ± 7 ng of the fusion protein/75 μg of chlorophyll of thylakoids (Fig. 4). This means that, since an average chlorophyll content per chloroplast is ~1 pg, a single chloroplast is able to accumulate 15,000 molecules of the i23K-BCCP-avidin complex occupying the import machinery for the ΔpH-dependent pathway. However, maximal import rates of only 700–900 molecules of proteins/one chloroplast/min have been reported for the ΔpH pathway (22, 26). This may mean that passage through the translocation pore is not a rate-limiting step in the ΔpH-dependent pathway across the thylakoid membranes, although we cannot rule out the possibility that a part of the 23K-BCCP molecules is not associated with the translocation pore.

The present study has revealed that the ΔpH-dependent pathway and the Sec-dependent pathway for thylakoidal protein transport do not converge at the translocation pores. This is consistent with the observation that the translocation across the cytoplasmic membrane via the Tat system in E. coli, a

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2. T. Nohara, unpublished data.
translocation pathway related to the thylakoidal ΔpH pathway, is independent of SecY and SecE (20). In analogy to the bacterial Sec system, the translocation pore for the Sec pathway likely consists of cpSecY/cpSecE and unidentified other chloroplast homologues of Sec proteins. The subunits constituting the translocation pore for the ΔpH-dependent pathway have not been identified yet, but Hcf106 is at least functionally linked to the pore. The successful generation of the translocation intermediate lodged in the thylakoid membranes will provide a valuable tool to identify components of the translocation pore for the ΔpH-dependent pathway in the future study.

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FIG. 4. Estimation of the number of import sites for the ΔpH-dependent pathway. A, increasing amounts of biotinylated i23K-BCCP were incubated with isolated thylakoids (75 μg of chlorophyll) in the presence of 2.5-fold excess weight of avidin. Halves of the reactions were treated with thermolysin (even-numbered lanes). B, amounts of translocation intermediate were estimated by quantifying m23K-BCCP digested by externally added thermolysin and are plotted against the amounts of i23K-BCCP input. Inset, a Scatchard-type plot of the same data for determination of the saturating amount of the translocation intermediate.