A Folded Protein Can Be Transported Across the Chloroplast Envelope and Thylakoid Membranes

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Many thylakoid luminal proteins are nuclear encoded, cytosolically synthesized, and reach their functional location after posttranslational targeting across two chloroplast envelope membranes and the thylakoid membrane via proteinaceous transport systems. To study whether these transmembrane transport machineries can translocate folded structures, we overexpressed the 17-kDa subunit of the oxygen-evolving complex of photosystem II (prOE17) that had been modified to contain a unique C-terminal cysteine. This allowed us to chemically link a terminal 6.5-kDa bovine pancreatic trypsin inhibitor (BPTI) moiety to prOE17 to create the chimeric protein prOE17-BPTI. Redox reagents and an irreversible sulfhydryl-specific cross-linker, bis-maleimidohexane, were used to manipulate the structure of BPTI. Import of prOE17-BPTI into isolated chloroplasts and thylakoids demonstrates that the small tightly folded BPTI domain is carried across both the chloroplast envelopes and the ΔpH-dependent transmembrane transporter of the thylakoid membrane when linked to the correctly targeted OE17 precursor. Transport proceeded even when the BPTI moiety was internally cross-linked into a protease-resistant form. These data indicate that unfolding is not a ubiquitous requirement for protein translocation and that at least some domains of targeted proteins can maintain a nonlinear structure during their translocation into and within chloroplasts.

INTRODUCTION

The outer and inner envelopes and the thylakoids constitute three membrane systems in chloroplasts. The envelope membranes are believed to contain one translocation system across which all nuclear-encoded internal chloroplast proteins are carried (Theg and Scott, 1993; Gray and Row, 1995). Distinct operationally independent protein transport machineries are present in each envelope membrane (Scott and Theg, 1996). Thylakoid luminal proteins encoded by the nucleus are synthesized in the cytosol as precursors containing a bipartite N-terminal extension that governs their posttranslational translocation across both the double-membrane chloroplast envelope and the thylakoid membrane. The N-terminal stromal-targeting domain specifies translocation across the chloroplast envelope membranes and is subsequently removed by a site-specific stromal processing peptidase. The resulting intermediate form is then transported across the thylakoid membrane into the lumen via one of two energetically distinct pathways by means of the lumen-targeting domain. Maturation is effected by a lumen-facing thylakoid membrane-bound processing peptidase that results in the appearance of a smaller mature protein within the thylakoid lumen (Hageman et al., 1986; Shackleton and Robinson, 1991). Transthylakoid translocation mechanisms that have so far been identified are believed to each be specific for a subset of luminal nuclear-encoded proteins. The isolation and characterization of chloroplastic homologues of bacterial SecA (Nakai et al., 1994; Yuan et al., 1994) has conclusively shown that one of these mechanisms is related to the bacterial Sec system. This translocation system is primarily responsible for the transport into the lumen of, among other proteins, plastocyanin (PC) and the 33-kDa subunit of the oxygen-evolving complex (OE33). Translocation via this pathway requires

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both nucleoside triphosphates and the chloroplastic SecA (Yuan et al., 1994). A transthylakoidal ΔpH is not required for this process but has been shown to stimulate the rate of translocation (Cline et al., 1992). Conversely, a subset of luminal proteins that include the oxygen-evolving complex subunits of 23-kDa (OE23) and 17-kDa (OE17; Cline et al., 1992; Klosgen et al., 1992), photosystem I subunit N (Nielsen et al., 1994), and photosystem II subunit T (Henry et al., 1994) do not require either nucleotide triphosphates or a stromal factor to be translocated across the thylakoid membrane. These proteins are transported by a mechanism that is supported solely by a ΔpH (Cline et al., 1992). The separation of these pathways has been elegantly demonstrated by in organello competition studies showing that high levels of iOE33 and iOE23 inhibit the transthylakoid transport of iPC and iOE17, respectively, but that these two groups do not cross-compete (Cline et al., 1993).

For a precursor protein to be translocated across the membrane of many organelles, it is thought to at least temporarily assume a conformation that differs from that of the stably folded mature protein (Schatz and Dobberstein, 1996). The requirement for such a conformational change was first demonstrated for mitochondrial protein import by an artificial precursor constructed by placing a yeast mitochondrial presequence in front of mouse dihydrofolate reductase (DHFR; Eilers and Schatz, 1986). Binding of methotrexate (MTX), which stabilizes the tertiary structure of the DHFR moiety, blocked import of the chimera across the mitochondrial membrane. Similar results have also been obtained in studies of protein transport across the plasma membrane of *Escherichia coli* (Wolfe and Wickner, 1984; Schiebel et al., 1991) and the endoplasmic reticulum (Muller and Zimmermann, 1988). These studies have used chimeric proteins that consist of a transport-competent targeting domain linked to a stably folded domain; import is thought to be completely or partially inhibited by the folded structure. Unfolding of the introduced C-terminal domain by removal of a bound ligand, such as in the DHFR-methotrexate MTX system (Eilers and Schatz, 1986), solubilization with urea (Eilers et al., 1988), or reduction of disulfide bonds of bovine pancreatic trypsin inhibitor (BPTI; Jascur et al., 1992) allows for the halted translocation intermediate to resume transport and subsequently become localized to its targeted compartment. Using such a trapped intermediate, Rassow et al. (1990) demonstrated that 50 amino acids were sufficient to span both the inner and outer membranes of mitochondria. The conclusion from these studies is that polypeptides generally traverse biological membranes after adopting loosely folded or even unfolded extended conformations.

A linear conformation, however, does not appear to be a requirement for translocation of proteins into all organelles. The nuclear transport machinery is known to be able to transport molecules as large as 23 nm (Feldherr et al., 1984; Dworetzky et al., 1988). Recently, Walton et al. (1995) clearly demonstrated that prefolded proteins stabilized with disulfide bonds and chemical cross-linkers can act as substrates for peroxisomal import. These authors also established that colloidal gold particles 4–9 nm in diameter conjugated to proteins bearing a C-terminal S-K-L peroxisomal targeting signal could traverse the peroxisomal membrane.

Construction of chimeric chloroplastic precursors has revealed that inhibition of transenvelope translocation can be achieved by the presence of introduced C-terminal domains. Wu et al. (1994) inhibited translocation of a precursor-DHFR construct crossing the chloroplast envelope by prebinding antibodies to the C-terminal DHFR domain. Schnell and Blobel (1993) also slowed envelope translocation of proteins by incorporating the IgG-binding domain of protein A into the C terminus of the precursor. Addition of IgG prior to the initiation of import halted the translocation process due to formation of a bulky protein-IgG complex. These studies clearly demonstrate that the chloroplast envelope translocation machinery can be arrested by the presence of a sufficiently large and folded protein domain. Further evidence of a conformational requirement for chloroplast protein import has come from the study of the transport and assembly of various holoenzymes. della-Cioppa and Kishore (1988) found that stabilization of the tertiary structure of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) by formation of a ternary complex with shikimate 3-phosphate and glyophosate inhibited transport of preEPSPS into the chloroplast stroma. This inhibition appeared to be due to interference with the membrane translocation step and not to an interaction with receptor binding or stromal processing. Similarly, Reinbothe et al. (1995) showed that binding of chlorophyllide (chlide) to precursor NADPH:protochlorophyllide oxidoreductase (POR) apparently stabilized POR in a transport-incompetent form, although this effect was not observed upon binding of protochlorophyllide or NADPH to POR.

In contrast to these studies, binding of MTX to DHFR did not halt translocation of a precursor traveling across the chloroplast envelope (Guera et al., 1993; America et al., 1994). Using protease digestion assays, Guera et al. (1993) concluded that this was due to unfolding of the DHFR domain at the chloroplast envelope accompanied by release of the MTX ligand, followed by penetration of MTX across the envelope and rebinding to the newly imported DHFR-precursor protein in the stroma. These data suggest that there are significant differences in the capabilities of the chloroplast envelope and mitochondrial membranes.
for unfolding and translocating tightly folded structures.

The substrate conformation required for the translocation machineries of the thylakoid membrane has not been as extensively investigated. Endo et al. (1994) showed that a C-terminal MTX-bound DHFR domain can halt the translocation of PC on the Sec-related thylakoid pathway. However, no arrested translocation intermediates have yet been generated in the ΔpH-dependent pathway. At present, apart from the energetics and substrate specificity, very little is known about the nature of this unique translocation mechanism. Creighton et al. (1995) demonstrated that the stromal intermediate form of OE23 is tightly folded, and we (Roffey and Theg, 1996) have suggested that efficient translocation of this protein requires that it be in a specific folded conformation for interaction with the thylakoid membrane. In this study, we have sought to determine whether the ΔpH-dependent pathway is sensitive to the presence of a folded protein domain by constructing a ΔpH-pathway-targeted precursor, prOE17, with a C-terminally located BPTI domain. We found that this chimeric protein was competent for transport across all chloroplast membranes, even when the BPTI moiety had been internally cross-linked to make unfolding impossible. The results demonstrate that chloroplasts can import and properly localize fully folded proteins.

**MATERIALS AND METHODS**

**Construction of the prOE17ΔG217ΔC Expression Vector**

A unique cysteine residue was engineered into the C terminus of the coding sequence of the maize prOE17 cDNA (Ettinger and Theg, 1992) by polymerase chain reaction (PCR)-directed mutagenesis. The modified precursor was constructed in several steps. First, a 130-bp fragment from positions −61 to +69 of the plasmid prOE17 was amplified by PCR. This allowed the introduction of an Ndel site just 5′ to the position of translational initiation. Second, a 190-bp fragment from positions 520 to 710 was amplified, which allowed the placement of a BamHI site at the C terminus and replacement of the C-terminal glycine with a unique cysteine residue. The entire coding region of the modified prOE17 was then reconstructed in the plasmid pGEM3 to create the plasmid pGEM3-prOE17ΔG217ΔC. The introduced changes were confirmed by DNA sequence analysis.

To generate sufficient quantities of the prOE17ΔG217ΔC precursor protein for chemical linkage studies, the prOE17ΔG217ΔC construct was inserted into the Ndel/BamHI sites behind the T7 promoter of the pET3a overexpression vector to form pET-prOE17ΔG217ΔC. The prOE17ΔG217ΔC-modified precursor was overexpressed in *E. coli* with the isopropyl β-D-thiogalactoside-inducible T7 promoter-based system in the presence of 1 mM/l [3H]leucine (150 Ci/mm). The overexpressed prOE17ΔG217ΔC was sequestered in inclusion bodies, thereby greatly simplifying its purification. The induction and purification of the inclusion body fraction was essentially as described by Cline et al. (1993) with the single modification that cells were lysed while on ice with a probe sonicator (Virtis Virasonic 300, The Virtis Company, New York, NY) using three 15-s pulses at 1-min intervals on a power setting of 3. The specific radioactivity of the precursor proteins was determined from the bicinchoninic acid protein assay and scintillation counting of inclusion bodies solubilized with 4% SDS. This method was verified by determining the specific radioactivity of the protein band on a gel by a combination of comparison to Coomassie blue-stained bovine serum albumin and fluorography of known standards. Specific radioactivities ranging from 4 × 10⁵ to 1.2 × 10⁶ dpm/mg protein were obtained with this method.

**Preparation of the Precursor Proteins**

BPTI was purchased as Aprotinin from Boehringer Mannheim (Indianapolis, IN) and maleimide derivatized with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as described by Vestweber and Schatz (1988). The resulting activated BPTI solution was adjusted to 6 mM urea and incubated at 20-fold molar excess with 40 mM prOE17 protein that had been previously solubilized from inclusion bodies in 2 ml of 6 M urea and 20 mM Na₂HPO₄ at a final pH of 7.0. The linkage reaction was carried out at room temperature for 30 min. Control reactions were prepared identically apart from the presence of 5 mM diethiothreitol (DTT) to prevent linkage between BPTI and prOE17. Reactions were adjusted to 8 M urea prior to import and checked for linkage efficiency by SDS-PAGE and fluorography. These reactant products were used directly in plastid protein import experiments.

**Protein Import Reaction Conditions**

Isolation of intact chloroplasts and preparation of thylakoids was performed as described by Ettinger and Theg (1991). Chloroplasts were resuspended in import buffer (IB) containing 330 mM sorbitol and 50 mM potassium-Tricine, pH 8.0. Thylakoids were resuspended in 1× IB supplemented with 5 mM MgCl₂. Import reactions were performed in 1× IB supplemented with 5 mM MgCl₂ and 5 mM ATP. Overexpressed precursors were added to a final concentration between 0.1 and 0.4 µM in a urea concentration of <0.2 M, giving between 6 × 10⁶ and 9.6 × 10⁶ dpm/60 µl of import reaction mixture. Import reactions were initiated by the addition of plastids to a final concentration of 0.33 mg of chlorophyll/ml within 1 min of the addition of precursors. After import under illumination, reactions were terminated by the addition of 3.3 mM HgCl₂ (Reed et al., 1990) or by dilution with ice-cold 1× IB and pelleting at 4000 rpm for 1 min in a microfuge at 4°C prior to further fractionations. Thermolysin digestions were performed for 30 min on ice. These were initiated by the addition of 0.1 volume of 2 mg/ml thermolysin in 1× IB and 100 mM CaCl₂ and halted by the addition of 0.1 volume of 250 mM EDTA in 1× IB. All fractionations of chloroplasts and thylakoids were carried out as described by Cline et al. (1992). Thylakoids were ruptured for separation into soluble and nonsoluble fractions with 0.1% Triton X-100 (Ettinger and Theg, 1991). Samples were analyzed by SDS-PAGE on 15% gels; gels were fluorographed and bands were quantitated with a Bioimage analysis system (Ettinger and Theg, 1992) after confirmation that fluorographic signals were in the linear range by comparison to standards.

**Treatment of Precursors with Ferricyanide and DTT**

BPTI-derivatized prOE17 reactions were diluted 1:10 with distilled water and incubated with either 20 mM DTT or 10 mM ferricyanide (FCN) at 57°C for 10 min or 2 min, respectively. Samples were mixed with 0.1 volume of 100% trichloroacetic acid (TCA) and incubated for 15 min on ice. Protein was collected by centrifugation at 100,000 × g for 15 min at 4°C. The resulting precipitate was washed with 100% acetone, dried, and then solubilized in the original volume of 8 M urea and 50 mM Tris-HCl at pH 8.0 for 4 h prior to use in import reactions.

**Alkylation Analysis for Available Sulfhydryls**

Analysis of BPTI and the prOE17-BPTI chimera for available sulfhydryls was carried out according to the protocol of Creighton (1980), with the following modifications. BPTI was analyzed after a
in the presence of tritiated leucine resulted in sequestration in inclusion bodies of radiolabeled prOE17, which was then purified, solubilized in urea, and imported into chloroplasts and thylakoids following the protocols of Cline et al. (1993). Cleavage of the transit peptide by the thylakoid processing peptidase during import into the lumen was observed, and the resultant mature form was found as a soluble protein in the thylakoid lumen. This localization is identical to that achieved with the in vitro-translated unmodified prOE17 protein (Ettinger and Theg, 1991). The inclusion body protein was covalently linked via the sole C-terminal cysteine to BPTI with the irreversible bifunctional cross-linker MBs according to the procedure of Vestweber and Schatz (1988). In most experiments, greater than 50% of the overexpressed prOE17 was converted to the BPTI-containing chimera prOE17-BPTI (Figure 1), resulting in a mixed precursor sample that was used directly in subsequent import experiments.

The prOE17-BPTI Chimera Is Imported into the Thylakoid Lumen via the ΔpH-dependent Pathway

Incubation of the mixed precursor sample (prOE17 and prOE17-BPTI) with thylakoids under normal import conditions resulted in the appearance of the expected 16.4-kDa luminal mature OE17 (mOE17) species (Figure 2, lane 2). Fractionation of the thylakoids after the import reaction demonstrated that a luminalized species of approximately 23 kDa was also present (Figure 2, lane 4). Cleavage of the transit peptide from the chimeric prOE17-BPTI precursor upon entry into the thylakoid lumen would be predicted to generate a mature protein with the BPTI moiety attached that has a calculated molecular mass of 22.9 kDa. This species is thus indistinguishable by SDS-PAGE from externally located prOE17 (no BPTI), which has a molecular mass of 23.1 kDa. However, fractionation experiments in conjunction with postimport thermolysin treatment, which has been shown to eliminate all of the protein species outside the thylakoid membrane but not to penetrate into the luminal space (Cline et al., 1992), clearly demonstrates that this approximately 23-kDa species was colocализed with the 16.4-kDa mature OE17 in a protease-impermeable soluble compartment (Figure 2, lanes 5 and 7).

The low detergent concentration we used for thylakoid lysis has been demonstrated to be below that necessary to release chlorophyll from the thylakoid membranes (Ettinger and Theg, 1991), and, therefore, it is unlikely that this result is due to disruption of membrane integrity and release of a membrane intermediate. We sometimes noted that a small amount of the 23-kDa species fractionated with the thylakoid membrane. Although this could indicate that a small percentage of this protein is trapped within the mem-
brane, the vast majority of it fractionated with the soluble lumen constituents.

The underivatized protein in the precursor preparation served as a convenient internal marker to follow the final compartmentalization of the prOE17-BPTI protein. The presence of this protease-protected species within the soluble compartment of the thylakoid suggests that it is not the unprocessed precursor, but rather represents the mOE17-BPTI species that has been fully translocated across the thylakoid membrane. Interaction of a stromal factor is not a requirement for this translocation as import of the chimera occurred in isolated thylakoids, as is also the case with prOE17. Similar experiments performed with intact chloroplasts revealed that prOE17-BPTI was translocated across the envelope as well as the thylakoid membranes (see below).

To establish whether the transport of prOE17-BPTI occurred via the transthylakoidal pathway normally used by prOE17, the kinetics and energetics of the import of the chimeric precursor were studied. A time course of protein import into intact chloroplasts is shown in Figure 3. To ensure rapid and complete halting of the reaction at the chosen time points, reactions were halted by the addition of HgCl₂, a poison introduced by Reed et al. (1990). When these samples were subsequently treated with thermolysin, the precursor forms were completely digested (Figure 3, t = 0), indicating that they were external to the chloroplast and that the protease efficiently degraded the precursor under these reaction conditions. After an initial lag period, the time course of import of both wild-type and chimeric species was generally linear over approximately 16 min. It is noteworthy that the accumulation of the mOE17 derived from the mixed precursor population did not seem to be affected by the presence of the prOE17-BPTI form in the import reaction, because its rate of import is identical to the control rate of prOE17 import shown in Figure 3A. However, the accumulation of the stromally located intermediate-sized species was affected, with the amount of this intermediate of each form being substantially lower than that observed when import was carried out with the single precursor. This may be indicative of some competition of the two forms for entry into the translocation pore of the chloroplast envelope at the concentrations used in this experiment [0.4 μM; compare Cline et al. (1993)]. The decrease in the amount of intermediate seen at later time points was the result of chloroplast lysis due to the long combined incubation times of the import reaction and the thermolysin digestion (30 min) required to completely remove the chimeric precursor protein, as this effect was not seen when the protease digestion step was omitted. At the 2-, 4-, and 6-min points, small amounts of the protease-protected precursor form reported by Scott and Theg (1996) were clearly visible (Figure 3A). This intermediate was also generated from the prOE17-BPTI form. It was not possible to distinguish between the protease-protected prOE17 species and the comigrating luminaly located mature OE17-BPTI in this experiment, with the result that the mature prOE17-BPTI (23-kDa band) form artifactualy appeared to accumulate rapidly during the first 4 min of the import reaction. The time-course experiment showed the rate of import across the chloroplast envelope and the thylakoid membrane, as seen by the appearance of the intermediate and mature forms, respectively, was similar (within a factor of approximately 2) for both the chimeric precursor and the underivatized prOE17 (Figure 3B).

A transmembrane pH gradient is the only energetic requirement for traversal of the thylakoid membrane by prOE17 (Cline et al., 1992). We examined whether a ΔpH was similarly necessary for the appearance of the mOE17-BPTI form within the thylakoid lumen. Elimination of the ΔpH was achieved by pretreatment of thylakoids with the ionophores nigericin and valinomycin. This combination of ionophores eliminates both components of the proton motive force (ΔpH and Δψ) and completely halts translocation of prOE17 into the thylakoid lumen (Cline et al., 1992; Figure 4, lanes 13 and 14). Import energetic requirements of the over-expressed protein were identical to that produced by the in vitro-translated precursor (Figure 4, lanes 6, 9, 12, and 15). When the BPTI-derivatized prOE17 was incubated with thylakoids in the presence of nigericin and valinomycin, no mature product was formed (Figure 4, lanes 10–12), indicating that no translocation had taken place. In the presence of the ionophores,
post-import thermolysin treatment removed all protein species of approximately 23 kDa (Figure 4, lanes 13–15) establishing that under these conditions this species consisted entirely of protease-sensitive precursor OE17 that remained external to the thylakoids. Clearly, the BPTI-derivatized mature protein was not formed in the absence of the ΔpH. The band just below the mOE17 band seen in several of the samples containing overexpressed protein (Figure 4, lanes 4 and 10) represents a contaminant present in the inclusion body preparation (Figure 4, lanes 1 and 2) and is not the mature protein; it was completely degraded by external thermolysin (Figure 4, lanes 7, 8, 13, and 14).

Thus, these data show that the kinetics and energetics of the translocation of the chimeric prOE17-BPTI are comparable to that of the prOE17 protein, establishing that both species followed the same pathway across the thylakoid membrane using the energy contained in the transmembrane ΔpH.

**The Folded State of the BPTI Domain Does Not Inhibit Translocation**

The folded structure of BPTI has been thoroughly investigated. The native state of this 58-residue monomeric protein is stabilized by the presence of three disulfide bonds between residues 14 and 38, 5 and 55, and 30 and 51, positions that dictate that the oxidized protein has a rather compact conformation (Creighton, 1975). BPTI is extremely stable in the native conformation with the folded state having a free energy 22.6 kJ/mol lower than the unfolded fully reduced form (Creighton and Goldberg, 1984). These features have allowed BPTI to be successfully used to halt translocation of attached precursor proteins across the mitochondrial membranes (Vestweber and Schatz, 1988) and across the *E. coli* plasma membrane (Schiebel et al., 1991). However, as has been shown by the experiments described above, BPTI did not inhibit the translocation of an attached precursor as it crossed the envelope or thylakoid membranes. One possible explanation for this result is that the BPTI domain somehow became reduced by the chloroplast membrane transport machineries, potentially allowing it to adopt an unfolded conformation prior to translocation.

The folded state of the precursor was investigated with the acid gel shift analysis developed by Creighton (1980) for BPTI. This procedure tests for available sulphydryls using alkylation with IAA, relying on the
charge alteration incurred by reaction of the thiol groups with IAA; the reduced protein in which all six thiol groups are reacted with IAA migrates electrophoretically more slowly toward a positive electrode than the native fully folded form (Creighton, 1980). This procedure was adapted for use with the prOE17-BPTI chimera and confirmed that the prOE17-BPTI was completely unfolded after the linkage reaction and before the import step. To further document that the BPTI moiety was not in an unfolded state prior to import, BPTI-linked prOE17 was pretreated with the redox reagents DTT and FCN. To remove the reagent after treatment, the precursors were TCA precipitated, washed with acetone, dried, and resuspended in 8 M urea for 24 h prior to import. This eliminated the possibility that these reagents might directly affect the translocation reaction. Creighton (1977) demonstrated that acid precipitation of BPTI and resuspension in 8 M urea after FCN treatment does not affect covalent disulfide bonds, which we confirmed by IAA treatment and electrophoretic analysis on acid native PAGE. In such a test, the DTT-pretreated prOE17-BPTI underwent a significant mobility shift after reaction with IAA, whereas the FCN-pretreated precursor did not. Import of these pretreated precursors into thylakoids followed by fractionation of the plastids revealed no appreciable difference in the amount of mature protein accumulated over a 20-min time course as a protease-resistant species in the thylakoid lumen (Figure 5). Lysis of the thylakoids with Triton X-100 followed by thermolysin treatment (Figure 5, lanes 6 and 7) showed that the mature species were thermolysin resistant only by virtue of their location and were not intrinsically protease resistant regardless of their conformation prior to import. The FCN and DTT treatments did not affect translocation of prOE17 in control reactions (Figure 5A). The faint band visible in lane 6 was also protease sensitive, as it was shown in other experiments to be completely digestible when exposed to thermolysin for longer incubations and probably represents a partial degradation product. This experimental finding indicates that the conformation of the C-terminally located BPTI domain prior to the import reaction is not a determinant for the ability of the protein to act as a substrate for the ΔpH-dependent translocation machinery. Furthermore, there was no significant decrease in the amount of mature OE17-BPTI protein present in the lumen by the end of a 20-min reaction, indicating that the folded BPTI domain did not require additional time to traverse the membrane as might be expected if the protein was unfolded during the transport step. Thus, these data suggest that unfolding of the BPTI moiety prior to import is not a requirement for translocation of the chimeric precursor.

Figure 4. Dissipation of the transmembrane pH gradient halts translocation of prOE17-BPTI into the thylakoid lumen. Transport reactions with overexpressed precursors were as described for Figure 2. In vitro-translated protein (lanes i.v.) was produced by transcription from the pET3a-prOE17G217AC plasmid with T7 polymerase and subsequent translation of the transcripts in the presence of 1 mCi/ml [3H]leucine. In vitro-translated precursors were added to import reactions to the same number of counts per milliliter as overexpressed protein and were supplemented with urea to the same concentration. Treatments with nigericin/valinomycin (N/V) were carried out by preincubation of plastids for 5 min with each ionophore (6 μM), and their final concentrations during the import reactions was 2 μM. Post-import thermolysin treatments were as described for Figure 3. Samples were analyzed by fluorography. Labels are as described in the legend of Figure 2.

Internally Cross-Linked BPTI Can Be Translocated into the Thylakoid Lumen

The experiments described above do not completely rule out the possibility that the BPTI moiety is unfolded prior to transport across the chloroplast membranes. In an effort to make unfolding impossible, we used a sulfhydryl-specific homobifunctional cross-linker, BMH, to covalently and irreversibly cross-link cysteine residues of BPTI to one another. Conditions were optimized for intramolecular cross-linking of the BPTI monomer prior to its attachment to the overexpressed prOE17. The BMH-cross-linked BPTI had a conformation different from native BPTI because it was slightly larger than the functional protein and no longer possessed trypsin inhibitor activity. BPTI samples were analyzed after BMH treatment for the completeness of the cross-linking reaction by gel shift analysis after alkalization with IAA (Figure 6). After IAA treatment, control BPTI samples that had been treated in an identical manner to the cross-linked samples, apart from the addition of the BMH, migrated partially as the native molecule and partially as the slower-migrating reduced form (Figure 6, lane 2). This is consistent with the data of Creighton (1977) in which it was shown that refolding of BPTI in 8 M urea results in one-quarter of the BPTI molecules regaining a native-like conformation. In contrast to this, IAA treatment of the BMH-cross-linked protein did not result in a mobility change. Addition of the BMH cross-linker resulted in all of the BMH-treated protein migrating at a mobility that was slightly less than that of the fully folded BPTI, which is likely due to the molecular weight change resulting from the binding of BMH.
BPTI was completely resistant to both thermolysin and trypsin over the entire time course examined, and none of the degradation products apparent after digestion of control BPTI were visible in the reactions with the BMH-cross-linked protein. BMH-treated BPTI still remained fully resistant to proteolytic degradation after an additional 30-min treatment with 20 mM DTT. This protease resistance was not due to the activity of BPTI, as it was observed with thermolysin as well, and the inhibitor activity was lost upon BMH cross-linking. We concluded, therefore, that the BMH treatment formed covalent and irreversible intramolecular cross-links between the six cysteine residues of BPTI, resulting in a protease-resistant protein with considerable tertiary structure.

The BMH-modified BPTI was linked to prOE17 according to the procedure described previously. This chimeric precursor, which now contained an irreversibly cross-linked C-terminal domain, was presented to chloroplasts under standard import conditions. Import of the chimeric precursor into chloroplasts resulted in the appearance of a mature OE17-BPTI form that was entirely resistant to external thermolysin treatment (Figure 5B, lane 3). It is noteworthy that the radiolabel was in the OE17 portion of the chimera, and therefore the protease resistance of the chimera observed in this experiment is unrelated to the protease resistance of the BPTI moiety described for the experiment represented in Figure 7. Fractionation of the chloroplasts after thermolysin treatment demonstrated that this mOE17-BPTI protein was localized within the thylakoid lumen (Figure 5B, lane 7). The localization of the prOE17 linked to BMH-cross-linked BPTI after import in chloroplasts was indistinguishable from that of the precursor attached to the un-
File 6. BMH-cross-linked BPTI is in a nonreducible state. BPTI was internally cross-linked with BMH as described in MATERIALS AND METHODS. Reducibility of samples was tested by treatment with 10 mM DTT followed by alkylation with 50 mM IAA for 10 min at room temperature. Samples were analyzed by 8 M urea, 18% acrylamide acid native gels as described in MATERIALS AND METHODS. Proteins were visualized using Western blotting and chemiluminescent detection. R, reduced BPTI; N, native BPTI.

treated BPTI. These data demonstrate that the BPTI moiety can be translocated across both the chloroplast envelope and the thylakoid membrane in a fully folded conformation.

The same precursors were presented to isolated thylakoids to determine whether the absence of components in the stroma would affect the translocation of the BMH-treated prOE17-BPTI chimera across the thylakoid membrane. Subsequent fractionation revealed that a mOE17-BPTI form colocalized in the thylakoid lumen with the native mature OE17. External thermolysin treatment of the intact thylakoids demonstrated that this form had fully crossed the thylakoid membrane. Import reactions carried out in intact chloroplasts with the precursor to which the BMH-cross-linked BPTI had been attached also resulted in the appearance of a mature OE17-BPTI species that cofractionated with the mature OE17 (our unpublished results). This protein was in a thermolysin-insensitive compartment and was present in amounts similar to those of the non-BMH-cross-linked form. These data indicate that the presence of the BMH-cross-linked BPTI domain did not inhibit the translocation of this precursor across the thylakoid membrane and that no soluble stromal components were necessary for the translocation to proceed. From these results we conclude that the internally cross-linked BPTI domain, when linked to a translocating precursor, can be conveyed by the ΔpH-dependent translocation machinery into the thylakoid lumen while retaining a considerable amount of tertiary structure.

DISCUSSION

In this study, we examined the chloroplastic targeting of a synthetic chimeric precursor consisting of an authentic precursor protein linearly linked to a tightly folded protein domain. We have provided kinetic and energetic evidence that this construct is translocated into the thylakoid lumen via the normal pathway followed by the native precursor. We have further shown that it is capable of being carried across both the chloroplast envelope and the thylakoid membranes even when the C-terminal BPTI domain had been chemically treated to retain it in a folded protease-resistant conformation. The data clearly demonstrate that unfolding of this conformationally stable domain is not a requirement for transfer through the chloroplast envelope and thylakoid membrane translocation machineries.

Previous investigators have generated trapped translocation intermediates in the chloroplast envelope by using folded domains to impede transfer. Schnell and Blobel (1993) identified two envelope-spanning import intermediates formed with an engineered chloroplast precursor protein by binding IgG to the C-terminal IgG-binding domain of staphylococcal protein A linked to the targeting domain of the precursor to the small subunit of Rubisco. Wu et al. (1994) also trapped a protein within the membrane during trans-envelope translocation by preincubating it with antibodies specific to its C terminus. This strategy also proved to be successful for Alefsen et al. (1994). However, not all attempts to generate translocation intermediates with chimeric constructs have been successful. Transport of a ferredoxin-DHFR fusion protein across the chloroplast envelopes was not
translocation of the tightly folded BPTI domain but not IgG.

Passage of BMH-treated prOE17-BPTI via the ΔpH-dependent transport machinery of the thylakoid membrane might similarly be due to the relative small size of BPTI. It is impossible to estimate the size limit for this transporter, since no translocation intermediates have been trapped crossing this pathway to date. We note that while Endo et al. (1994) found that a full-length PC molecule was halted in its transfer across the thylakoid membrane by the presence of a DHFR-MTX C-terminal domain, this protein is transported via the so-called Sec-related pathway and likely traverses the membrane through a pore similar to the 20–60-Å channel formed by Sec61p (Hamman and Johnson, 1996; Hanein et al., 1996). In contrast to this, Creigton et al. (1995) recently investigated the structure of the stromal intermediate of the oxygen-evolving 23-kDa subunit (OE23), a substrate of the ΔpH-dependent pathway. This soluble transient form contained a protease resistant core of 22 kDa that was insensitive to trypsin digestion in an aqueous environment but was rapidly digested once unfolded by urea or heat treatments. These authors proposed that this protein must, therefore, interact with the thylakoid receptors while in a folded state and suggested that the lack of a requirement for a soluble stromal factor implied that no preimport unfolding is necessary. Accordingly, they concluded that the iOE23 protein must become unfolded during translocation. Recent work from our laboratory has shown that alteration of the conformation of the prOE23 by deletion of small C-terminal domains leads to inefficient thylakoid transport and rapid degradation in the thylakoid lumen, suggesting that the normal folded conformation is necessary both for recognition of the substrate by the transporter and for stability in the lumen (Roffey and Theg, 1996). In light of our current experiments showing that the overexpressed prOE17-BPTI chimera was transported across the thylakoid membrane in a completely folded and chemically cross-linked state in the absence of any stromal factors, the combined data indicated that a linear conformation is not an absolute requirement for substrates crossing the ΔpH-dependent pathway.

The ability of certain translocators to transport folded structures is not without precedent. Glover et al. (1994) found that Saccharomyces cerevisiae peroxisomal thiolase dimers can be imported without disruption of the association between the two subunits. Proteins containing disulfide bridges have been shown to act as substrates for the outer and inner membranes of Vibrio (Hardie et al., 1995), and chemically cross-linked nonlinear molecules have been demonstrated to be translocated into everted E. coli membrane vesicles in the presence of a proton motive force (Tani and Mizushima, 1991). Recently, Walton et al. (1995) showed

Figure 8. BMH-cross-linked prOE17-BPTI is transported across the chloroplast envelopes and into the thylakoid lumen in intact chloroplasts. Precursors were presented to intact chloroplasts as described for Figure 3. Chloroplasts were fractionated after thermo-
lysin treatment, and samples were analyzed by SDS-PAGE and fluorography. T.M., membrane samples; T.L., thylakoid lumen. Other labels are as described in the legend of Figure 2.
that prefolded proteins stabilized with disulfide bonds and chemical cross-linkers remained substrates for peroxisomal import, as were mature folded and disulfide-bonded IgG molecules containing the peroxisomal targeting signal. Furthermore, 4–9-nm colloidal gold particles conjugated to proteins bearing a peroxisomal targeting signal were translocated into the peroxisomal matrix. These authors speculated that a pore complex, not unlike that of the nuclear envelope, may be present in peroxisomal membranes that would allow for the transport of folded structures. It is difficult to imagine such an open structure existing in the thylakoid membrane without compromising their role in energy transduction, and evidence from our laboratory indicates that this is not the case (Teter and Theg, unpublished data). Alternatively, some form of endocytosis of the membrane could be suggested; however, such a mechanism raises questions about the fate of the invaginated membranes and the mechanism of release of the vesicular contents. The remaining possibility is that the plastid translocation machineries studied herein (envelope and the ΔpH-dependent thylakoid pathways) have sufficient flexibility to allow the uptake of a domain at least 2.3 nm in diameter. This process must occur in such a way that it does not threaten the ability of thylakoids to maintain a transmembrane ion gradient, as translocation presumably occurs in fully functional photosynthetically active organelles. The details of the mechanism used by chloroplasts to translocate such structures across its membranes remain to be elucidated.

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