The Hydrophilic Domain of Tic110, an Inner Envelope Membrane Component of the Chloroplastic Protein Translocation Apparatus, Faces the Stromal Compartment*

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The majority of chloroplastic proteins are encoded within the nuclei of plant cells and are synthesized on cytoplasmic ribosomes. As a result, these proteins must be imported into the chloroplast post-translationally, usually via an unfolded, higher molecular weight precursor form containing a N-terminal transit peptide (1–4). Once a precursor protein has entered the chloroplast stroma, the transit peptide is cleaved off by the stromal processing peptidase, and the protein is folded into its mature form (4). Protein import into chloroplasts is mediated by a proteinaceous translocation apparatus and components of the outer membrane translocation apparatus (13, 14). Tic110 is an integral protein of the inner envelope membrane of chloroplasts, with either one or two putative, hydrophilic, transmembrane domains located near its N terminus (13, 14). The overall topology of Tic110 within the inner envelope membrane, however, remains a point of debate. Lübeck et al. (14) reported that Tic110 spans the membrane once and that its large (>90-kDa) hydrophilic domain is oriented toward the intermembrane space between the outer and inner envelope membranes. On the other hand, Kessler and Blobel (13) proposed that Tic110 spans the membrane twice and that its hydrophilic domain is contained within the chloroplast stroma. To date, no evidence has been presented that satisfactorily resolves this controversy.

Knowing the topology of Tic110 will be important in assigning a putative function to this protein. For instance, if Tic110 is oriented toward the chloroplast intermembrane space, it may function by interacting with the outer membrane translocation apparatus, promoting the formation of contact sites between the two envelope membranes (14). However, if Tic110 is instead exposed to the stromal compartment, then it is more likely that the protein acts by recruiting stromal proteins, for example molecular chaperones, to the translocation apparatus during protein import.

The first component of the inner envelope membrane translocation apparatus to be cloned was Tic110. Using chemical cross-linking and coimmunoprecipitation techniques, two separate laboratories have found Tic110 in a complex with both a translocating precursor and components of the outer membrane translocation apparatus (13, 14). Tic110 is an integral protein of the inner envelope membrane of chloroplasts, and its large (>90-kDa) hydrophilic domain is oriented toward the intermembrane space between the outer and inner envelope membranes. On the other hand, Kessler and Blobel (13) proposed that Tic110 spans the membrane twice and that its hydrophilic domain is contained within the chloroplast stroma. To date, no evidence has been presented that satisfactorily resolves this controversy.

In previous studies, the topology of Tic110 was investigated by analyzing the protease sensitivity of the protein within intact chloroplasts, a technique that has been used for other chloroplastic membrane proteins and for membrane proteins of other organelles (7, 11, 13, 14, 17, 18). Two of the most widely used proteases in such studies are thermolysin and trypsin. Thermolysin has been used to selectively degrade outer envelope membrane proteins exposed on the surface of chloroplasts, since this protease, at moderate concentrations, does not penetrate the outer membrane (19). Trypsin, however, does penetrate the chloroplast outer envelope membrane, but it does not, at moderate concentrations, destroy the permeability barrier of the inner membrane (19–21). Thus, trypsin is useful in defining the topology of inner envelope membrane proteins and in localizing soluble proteins to the intermembrane space of the chloroplast.

In this paper, we report on the topology of Tic110, attempting...
to resolve the controversy that currently exists concerning the orientation of this protein within the chloroplast inner envelope membrane. When steps are taken to adequately quench proteases, Tic110 is degraded by neither trypsin nor thermolysin, indicating that the large hydrophilic domain of Tic110 is contained within the chloroplast stromal compartment. In addition, when trypsin is insufficiently quenched, Tic110 is degraded, but only after chloroplasts are broken open. Comparison of the protease sensitivity of Tic110 with those of proteins of established topology lends further support to the conclusion that Tic110 is indeed oriented toward the chloroplast stroma.

**EXPERIMENTAL PROCEDURES**

**Materials**—Pea seeds (*Pisum sativum* var. *little marvel*) were supplied by the Olds Seed Company (Madison, WI). Percoll silica gel, trypsin (from bovine pancreas), N'-p-tosyl-L-lysine chloromethyl ketone (TLCK), soybean trypsin inhibitor, and Mg-ATP were obtained from Sigma. Phenylmethylsulfonyl fluoride (PMSF) and aprotinin were described by Tranel et al. (12). Antiserum to Tic110 was generated as described previously (12). Antiserum to Toc34 (9) was a gift from D. Schnell. Antiserum against Toc75 was raised as discussed by Antisev (15). Antiserum to Tic75 was raised as discussed by Travell et al. (12). Antiserum to Tic34 (9) was a gift from D. Schnell. Affinity-purified anti-ClpC antibodies (27) were a gift from J. Shanklin.

**RESULTS**

**Tic110 Is Resistant to Digestion by Adequately Quenched Trypsin**—It has been reported that certain proteases, most notably trypsin, are able to destroy the permeability barrier of the outer envelope membrane of chloroplasts and thereby degrade outer membrane proteins, as well as inner envelope membrane proteins exposed to the intermembrane space, while leaving stromally exposed proteins undigested (19–21). Consequently, this method can be used to selectively degrade inner envelope membrane proteins that are oriented toward the intermembrane space while leaving stromally exposed inner membrane proteins intact. Such selective proteolysis techniques have previously been utilized to analyze the location and topology of various chloroplast envelope membrane proteins, including Tic110 (13, 14, 18).

During efforts to repeat and extend these previous studies, we observed that Tic110 was resistant to degradation when intact chloroplasts were incubated with a range of trypsin concentrations (data not shown), indicating that this protein was not exposed to the chloroplast intermembrane space. These results were in direct contrast with the trypsin sensitivity of Tic110 reported by Lübeck et al. (14). However, several differences in protocol existed between the two experiments, including the length of time used for trypsin digestion and the reagents used to quench trypsin activity. Consequently, we sought to determine whether these protocol differences could explain the contrasting results.

Intact chloroplasts were incubated with trypsin for either 10 min (Fig. 1, lanes 1–3 and lanes 7–9) or for 60 min (Fig. 1, lanes 4 and 5 and lanes 10 and 11), as described by Lübeck et al. (14).

**Experimental Procedures**

**Isolation of Chloroplasts**—Chloroplasts were isolated from 8–12-day-old pea seedlings over Percoll gradients as described previously (22).

**Trypsin Digestion of Intact Chloroplasts**—Chloroplasts were isolated from 8–12-day-old pea seedlings over Percoll gradients as described previously (22). Final resuspension was in import buffer (50 mM HEPES-KOH (pH 8.0), 4 mM MgCl₂) at a concentration of 0.5 mg/ml chlorophyll. Protein concentration was determined by the Bradford protein assay. Purified intact chloroplasts (100 mg chlorophyll) in import buffer containing calcium chloride at a final concentration of 0.1 mM. The final reaction volume for these digestions was 300 ml. After incubation with the protease for either 10 min or 60 min at room temperature, trypsin activity was quenched by adding either PMSF at a final concentration of 1 mM or by adding a mixture of protease inhibitors to a final concentration of 1 mM PMSF, 0.05 mg/ml TLCK, 0.1 mg/ml soybean trypsin inhibitor, and 2 mg/ml aprotinin. Chloroplasts were incubated with the quenching reagents for 10 min on ice.

After quenching, intact chloroplasts were resuspended over a 40% (v/v) Percoll cushion. The recovered chloroplasts were lysed hypotonically and fractionated into crude membrane and soluble fractions as described previously (22), except that the lysis buffer contained either PMSF at a final concentration of 1 mM or a protease inhibitor mixture described previously (22), except that the lysis buffer contained either PMSF at a final concentration of 1 mM or a protease inhibitor mixture described previously (22). The protein concentration of each fraction was determined by the Bradford protein assay. Membrane and soluble protein fractions were analyzed by SDS-PAGE and immunoblotting with antibodies against either Tic110 or Toc75.
When 1 mM PMSF was used to quench trypsin activity. These observations indicated that 1 mM PMSF was insufficient to quench protease activity. This result was supported by the finding that when chloroplasts were incubated with 1 mM PMSF prior to trypsin addition, Tic110 was still completely degraded (Fig. 1, lane 12). Other differences (i.e. number of washes) between our protease digestion protocol and that of Lübeck et al. (14) were also tested for their effects on Tic110 degradation. However, none affected the pattern of Tic110 digestion (data not shown). We concluded, therefore, that the difference in results could be completely explained by differences in the methods used to quench trypsin activity.

Tic110 Is Degraded by Insufficiently Quenched Trypsin only after Chloroplast Lysis—We next sought to determine at what stage of the protease digestion protocol trypsin degraded Tic110 when 1 mM PMSF was used as the quenching reagent. Specifically, we wanted to determine whether Tic110 was degraded before chloroplast lysis, when the permeability barrier of the inner membrane was still intact, or after lysis, when the inner membrane had been ruptured. There were three stages during our protocol in which degradation of Tic110 by trypsin could occur: before chloroplasts were broken open (incubation of chloroplasts with trypsin, quenching of protease activity, and reisolation of intact chloroplasts), during chloroplast lysis, or during postlysis steps (membrane sedimentation and incubation of the membranes in SDS-PAGE sample buffer). To distinguish among these possibilities, we quenched trypsin-treated chloroplasts with the mixture of protease inhibitors before lysis, during lysis, and/or after lysis. During those steps when the protease inhibitor mixture was not added, 1 mM PMSF was added in its place.

Fig. 2 shows the results from this experimental approach. When the quench mixture was added at all three stages or just during and after lysis, Tic110 was not significantly degraded (Fig. 2A, lanes 1 and 2). Tic110 was completely digested only when the quenching mixture was added just during the postlysis stage (Fig. 2A, lane 3), indicating that it was most likely degraded by active trypsin during chloroplast lysis. In addition, as long as the protease inhibitor mixture was added before and/or during chloroplast lysis, Tic110 was not digested by trypsin (Fig. 2B). Thus, it appeared that unless trypsin was adequately quenched before or at the time of lysis, Tic110 was digested by the protease once chloroplasts were broken open.

Trypsin Degrades Proteins Exposed to the Intermembrane Space but Not Tic110—Recently, several investigators have utilized protease digestion techniques to analyze the location and topology of newly imported chloroplastic proteins (18, 23). We obtained these precursor constructs in order to determine whether the quenching protocol used had an effect on the results and to compare the protease sensitivity of constructs with known topology to that of Tic110 constructs. Intact chloroplasts were subjected to an import assay with one of five different precursor proteins: prToc75 (12); tp110–110N, a truncated version of prTic110 containing the putative transmembrane domain(s) and approximately one-fifth (<20 kDa) of the hydrophilic domain (23); tpSS-110N, a chimeric precursor containing the transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase (SS) attached to the truncated version of mature Tic110 (23); tpToc75-mSS, a chimeric precursor consisting of the transit peptide of Toc75 attached to the mature form of SS (18); and prSS (24). After import, intact chloroplasts were reisolated and digested with either thermolysin or trypsin. Trypsin-treated chloroplasts were quenched with either the protease inhibitor mixture (trypsin I protocol) or 1 mM PMSF (trypsin II protocol). Following protease digestion, intact chloroplasts were reisolated, lysed, and separated into membrane and soluble protein fractions. The proteins from these fractions were then analyzed by SDS-PAGE and fluorography to detect the newly imported, radiolabeled proteins (Fig. 3A) or immunoblotting to detect endogenous proteins (Fig. 3B).

The processed forms of prToc75 (mToc75 and iToc75), which were used as markers for the outer envelope membrane, were degraded by trypsin but not by thermolysin (Fig. 3A, row 1). Because Toc75 is deeply embedded in the chloroplast outer envelope membrane, thermolysin could not access the protein. However, because trypsin is able to penetrate the outer membrane, it was able to completely digest Toc75 (as in Fig. 1). In contrast, because neither thermolysin nor trypsin can penetrate the inner envelope membrane, prSS, a stromal marker, was not digested by either protease (Fig. 3A, row 5).

It has previously been reported that when tpToc75-mSS is imported into chloroplasts, the processed product is exposed to the chloroplast intermembrane space in both soluble and membrane-bound forms (18). Thus, we utilized this construct as a marker for the intermembrane space. Accordingly, we found that both the soluble and membrane-bound products generated from tpToc75-mSS were degraded by trypsin but not by thermolysin (Fig. 3A, row 4). If Tic110 was also exposed to the intermembrane space, we would have expected it to have a protease sensitivity similar to tpToc75-mSS. However, neither of the Tic110 constructs, tp110–110N and tpSS-110N, which were expected to have the same topology as Tic110 itself (23), were digested by trypsin as long as protease activity was sufficiently quenched by the protease inhibitor mixture (Fig. 3A, rows 2 and 3, compare trypsin I and trypsin II protocols). We interpreted these results to indicate that these Tic110 constructs were not exposed to the intermembrane space. Since it has been previously demonstrated that these two constructs are inserted in the inner envelope membrane (23), we con-
or with 1 mM PMSF (row 3), prToc75 (row 1), tpToc75–110N (row 2), tpSS–110N (row 4), and prSS (row 5) were imported into isolated chloroplasts (100 μg of chlorophyll). Intact chloroplasts were reisolated and divided into four equal samples. The four samples from each import reaction were protease-treated as indicated above and as outlined under “Experimental Procedures.” Trypsin-treated samples were quenched either with a mixture of protease inhibitors (Trypsin I) or with 1 mM PMSF (Trypsin II). Intact chloroplasts were reisolated from each sample, lysed, and separated into membrane (P) and soluble (S) protein fractions. Equivalent protein from each fraction was analyzed by SDS-PAGE and either fluorography (A) or immunoblotting (B) with antibodies against Tic110, Toc75, and Toc34.

FIG. 3. The trypsin sensitivity of newly imported Tic110 constructs does not mimic that of an intermembrane space marker protein. 35S-Labeled prToc75 (row 1), tp110–110N (row 2), tpSS–110N (row 3), tpToc75–mSS (row 4), and prSS (row 5) were imported into isolated chloroplasts (100 μg of chlorophyll). Intact chloroplasts were reisolated and divided into four equal samples. The four samples from each import reaction were protease-treated as indicated above and as outlined under “Experimental Procedures.” Trypsin-treated samples were quenched either with a mixture of protease inhibitors (Trypsin I) or with 1 mM PMSF (Trypsin II). Intact chloroplasts were reisolated from each sample, lysed, and separated into membrane (P) and soluble (S) protein fractions. Equivalent protein from each fraction was analyzed by SDS-PAGE and either fluorography (A) or immunoblotting (B) with antibodies against Tic110, Toc75, and Toc34.

cluded that they must be oriented toward the chloroplast stroma.

We also examined the protease sensitivity of three endogenous proteins in these chloroplasts (Fig. 3B). Tic110 was not significantly degraded by either thermolysin or trypsin as long as trypsin was adequately quenched (Fig. 3B, lanes 1–4). This is similar to the results obtained in our previous experiments and those seen for the imported Tic110 constructs. Toc75 was degraded by trypsin but not by thermolysin (Fig. 3B, lanes 5–8), consistent with the results obtained for imported Toc75. On the other hand, Toc34 was degraded by both proteases (Fig. 3B, lanes 9–12). These results are consistent with the fact that the cytosolic domain of Toc34 is exposed on the outer surface of chloroplasts (7, 11).

Tic110 Is Exposed on the Same Face of Inner Envelope Membrane Vesicles as ClpC, a Stroma-facing Protein.—The results presented above suggest that within intact chloroplasts, Tic110 is oriented toward the stromal compartment. In order to extend and confirm this conclusion, we analyzed the topology of Tic110 in a second system, isolated inner envelope membrane vesicles. Specifically, we compared the trypsin sensitivity of Tic110 to that of ClpC, a stromal hsp100 homologue. ClpC is primarily a soluble protein; however, it is known that a significant portion of the ClpC molecules in the chloroplast are associated with the stromal side of the inner envelope membrane (28, 29). Therefore, if Tic110 is indeed exposed on the stromal face of the inner envelope membrane, it should display the same trypsin sensitivity as ClpC. This indeed was what we observed upon analysis of inner membrane vesicles (Fig. 4). Both Tic110 and ClpC were resistant to degradation at low protease concentrations and susceptible at higher levels of trypsin. In addition, both proteins began to be significantly degraded at the same trypsin concentration (Fig. 4, A, lane 5, and B, lane 5), indicating that Tic110 and ClpC were exposed on the same side of the vesicles. Consequently, we concluded that Tic110, like ClpC, was oriented toward the chloroplast stroma.

DISCUSSION

To investigate the process of protein import into chloroplasts in detail, it will be necessary to study the translocation machineries of the outer and inner envelope membranes separately, as has been done for the mitochondrial protein import system (30). Mitochondria, like chloroplasts, are surrounded by an envelope composed of two separate membranes. Techniques have been developed to physically remove the mitochondrial outer envelope membrane so that inner envelope membrane proteins can be specifically analyzed (31, 32). Mitoplasts, mitochondria in which the outer membrane has been selectively ruptured and/or dissolved, can be generated either by subjecting intact mitochondria to osmotic shock treatment (31) or by treating them with digitonin (32). These two methods have been used successfully to study the location and topology of mitochondrial inner envelope membrane proteins and the mechanism of mitochondrial protein import (for example, see Refs. 33–38).

Similar techniques to selectively remove the outer membrane of chloroplast envelopes are not yet available. In lieu of
such approaches, investigations on chloroplast inner envelope membrane proteins have relied on the ability of certain proteases, specifically trypsin, to selectively destroy the permeability barrier of the outer membrane and degrade inner membrane proteins that are exposed to the intermembrane space while leaving stromally exposed proteins intact (19–21). This technique can thus be used to differentiate between an intermembrane space and a stromal localization for both soluble and membrane proteins (13, 14, 18, 23), as we have done in this study.

Two independent investigations have provided evidence indicating that Tic110 is a component of the chloroplast protein translocation apparatus localized in the inner envelope membrane (13, 14). However, no function for Tic110 during protein translocation has been clearly established. Elucidating the topology of Tic110, about which the original reports disagreed (13, 14), will be an important first step toward understanding the role of this protein in the import process. In this investigation, we have provided evidence indicating that the large (>90-kDa) hydrophilic domain of Tic110 was oriented toward the stromal compartment. Because the one or two predicted transmembrane domains of Tic110 are near the N terminus (within the first 10% of the mature protein), it is likely that the regions of Tic110 that are important for its function reside within the large hydrophilic domain, which we have localized.

Previous investigations have proposed that Tic110 may be involved in mediating the interaction between outer and inner envelope membrane translocation components during protein import (14). However, our evidence does not support this view. The stromal orientation of the major portion of Tic110 would probably not allow this protein to interact with outer envelope membrane proteins. Instead, it is more likely that Tic110 interacts with stromal components of the translocation apparatus. For instance, Tic110 may be involved in the recruitment of molecular chaperones, including ClpC, to the site of protein import.

This study has demonstrated that Tic110 is degraded by trypsin only when trypsin-treated chloroplasts are insufficiently quenched. Incomplete quenching of trypsin activity with PMSF is the most likely explanation for previous reports concluding, based on trypsin analysis, that Tic110 is degraded by the protease and thus is oriented toward the intermembrane space (14). An investigation reported by Kessler and Blobel (13) and Toc75 (an outer envelope membrane protein normally susceptible to trypsin action) are left largely intact (Fig. 1). Thus, we concluded that the protease inhibitor mixture used in this investigation was sufficient to quench trypsin activity. Such a mixture of inhibitors should be useful in studying the topology of membrane proteins or in any other investigations where analysis by trypsin digestion plays a pivotal role.

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