The Thylakoid Proton Gradient Promotes an Advanced Stage of Signal Peptide Binding Deep within the Tat Pathway Receptor Complex

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The thylakoid membranes of plant chloroplasts and the cytoplasmic membranes of eubacteria and some archaea possess parallel protein translocation systems. The well characterized Sec systems transport unfolded proteins using NTPs as the energy source. The Tat (twin arginine translocation) systems transport folded proteins across the thylakoid membrane of chloroplasts and the plasma membrane of most bacteria. Tat precursors are targeted by hydrophobic cleavable signal peptides with twin arginine (RR) motifs. Bacterial precursors possess an extended consensus, (S/T)RRXFLK, of which the two arginines and the phenylalanine are essential for efficient transport. Thylakoid Tat precursors possess twin arginines but lack the consensus phenylalanine. Here, we have characterized two stages of precursor binding to the thylakoid Tat signal peptide receptor, the 700-kDa cpTatC-Hcf106 complex. The OE17 precursor tOE17 binds to the receptor by RR-dependant electrostatic interactions and partially dissociates during blue native gel electrophoresis. In addition, the signal peptide of thylakoid-bound tOE17 is highly exposed to the membrane surface, as judged by accessibility to factor Xa of cleavage sites engineered into signal peptide flanking regions. By contrast, tOE17 containing a consensus phenylalanine in place of Val^20 (V - 20F) binds the receptor more strongly and is completely stable during blue native gel electrophoresis. Thylakoid bound V - 20F is also completely protected from factor Xa at the identical sites. This suggests that the signal peptide is buried deeply in the cpTatC-Hcf106 binding site. We further provide evidence that the proton gradient, which is required for translocation, induces a tighter interaction between tOE17 and the cpTat machinery, similar to that exhibited by V - 20F. This implies that translocation involves a very intimate association of the signal peptide with the receptor complex binding site.

The thylakoid membranes of plant chloroplasts and the cytoplasmic membranes of eubacteria and some archaea possess parallel protein translocation systems. The well characterized Sec systems transport unfolded proteins using NTPs as the energy source. The Tat (twin arginine translocation) systems are distinctive in that they transport proteins in a folded conformation, and they employ only the protonotive force as energy source (for the most recent reviews, see Refs. 1 - 4). Both Sec- and Tat-directed precursor proteins possess hydrophobic cleavable signal peptides, which direct targeting to the respective machineries. Tat signal peptides differ from the Sec signal peptides in that they contain an essential twin arginine motif immediately upstream of the hydrophobic core region. Bacterial Tat substrates possess an extended consensus sequence consisting of (S/T)RRXFLK (5). Only one of the known thylakoid Tat precursor protein possesses the extended consensus (6). Rather, logoplot analysis of known and predicted thylakoid Tat substrates suggests a weak consensus of RRX-Hyd-Hyd, where Hyd represents a hydrophobic amino acid (7).

Commonly, three components are required for Tat transport: cpTatC, Hcf106, and Tha4 in thylakoids and the orthologous TatC, TatB, and TatA, respectively, in bacteria. The thylakoid Tat system has been experimentally staged into several steps (8, 9); the precursor protein binds to a cpTatC-Hcf106 receptor complex, a Tha4 oligomer assembles with the precursor-receptor complex to form the putative translocase (10), and the precursor is transported into the lumen. After transport, Tha4 dissociates from the receptor complex, resetting the system for another round of transport.

Identification of precursor proteins, such as tOE17, a truncated variant of the OE17 precursor that bind stably to thylakoids in the absence of the proton gradient, has allowed characterization of the initial binding step (11). On the other hand, certain observations suggest that precursors bind to the receptor complex differently, possibly more tightly, under the energized conditions that exist during protein transport. First, several Tat pathway precursor proteins do not bind stably in the absence of a proton gradient but are nevertheless efficiently transported (11). For instance, the OE23 precursor binds reversibly to thylakoids in the absence of a proton gradient, and its association with the receptor has only been detected by chemical cross-linking (9, 11). Second, although 150 mM KCl prevents tOE17 binding to the receptor complex in the absence of the proton gradient, 150 mM KCl does not significantly impair transport of tOE17 across energized membranes (12). In this regard, a recent study identified modified tOE17 proteins

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2 The abbreviations used are: cpTat, chloroplast Tat; IB, import buffer; (Tmd)Phe, l-4’-[3-(trifluoromethyl)-3H-diazirin-3-yl]phenylalanine; BN-PAGE, blue native polyacrylamide gel electrophoresis; GFP, green fluorescent protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
that do bind more stably to the receptor complex. Translational insertion of the photoreactive phenylalanine analog (Tmd)Phe was used in cross-linking studies to fine map interactions between tOE17 and cpTat components (13). Unexpectedly, we found that most of the Tmd(Phe)-substituted precursors that cross-linked to cpTat components formed tighter associations with the receptor that were not disrupted by salt washes. Strikingly, the most abundant cross-linking product was formed between cpTatC and tOE17 with Tmd(Phe) in place of Val\(^{-20}\) (V \(-20\)Tmd(Phe)), the location of the bacterial consensus Phe. One model for the Tat system implies a strong interaction between the precursor and cpTatC (TatC), because it proposes that cpTatC pulls the precursor through a Tha4-facilitated opening (10, 14). Consistent with this idea, tOE17 cross-linked to cpTatC through the V \(-20\)Tmd(Phe) site could still be efficiently transported into the lumen (13).

In the present work, we have further characterized the precursor-receptor interactions of tOE17 and a phenylalanine-substituted tOE17 (V \(-20\)F), which exhibits a stronger interaction comparable with its Tmd(Phe) derivative. In particular, whereas tOE17 was primarily bound through electrostatic interactions, V \(-20\)F required both salt and urea solutions to be released from the membrane. The V \(-20\)F association with the receptor complex was also more stable to blue native PAGE (BN-PAGE) analysis. Finally, V \(-20\)F, when bound to thylakoids, was virtually inaccessible to the protease factor Xa at a variety of cleavage sites introduced near the signal peptide, whereas bound tOE17 was highly accessible to factor Xa at the analogous cleavage sites. In order to determine if binding of V \(-20\)F is similar to the interaction of precursors under the energized conditions that occur during transport, tOE17 was bound to thylakoids pretreated with \(\alpha\)Tha4 IgGs (to prevent translocation) and a proton gradient was applied. In the presence of the proton gradient, bound tOE17 was less susceptible to salt extraction and less accessible to factor Xa cleavage than in the absence of a proton gradient. These results support a model in which, at the moment of transport, the signal peptide of Tat precursors becomes more tightly associated with and deeply buried in the binding site of the cpTatC-Hcf106 receptor complex. These results will be discussed in the context of current models for translocation by the Tat translocase.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Mutagenesis**—DNA clones for the various precursors used in this study were constructed by PCR mutagenesis with the QuikChange™ mutagenesis kit from Stratagene and the clones for tOE17 and iOE17 as templates (15). DNA sequencing on both strands at the University of Florida Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Facility verified all constructs. Phenylalanine substitutions were obtained by replacing the relevant codons with TTT. Signal peptide clones were obtained by inserting the stop codon TAG at the respective positions 17 and 30 residues into the coding sequence of the OE17 mature domain. The clone for iOE17 IEGR\(_{11}\) was prepared by substituting the coding sequence of iOE17 MASAGDA with MASIEGRA in vitro translation products were incubated with apyrase for 10 min at 0 °C and then with an equivalent volume of washed thylakoid membranes and an equivalent volume of IB for 15 min in darkness at 0 °C. Thylakoid membranes were then recovered by centrifugation with a stoichiometric quantity of stomatal extract, 5 mM Mg-ATP, and 2 mM dithiothreitol and incubation for 15 min at 25 °C in 70 μmol/m²/s white light. Thylakoid membranes were recovered by centrifugation and analyzed by SDS-PAGE and fluoroxygraphy.

**Bound Precursor Extraction Assays**—Precursor-bound thylakoid membranes (25 μg of chlorophyll) were resuspended in 75 μl of the extraction solution (freshly prepared in 10 mM HEPES/KOH, pH 8.0) and incubated at 0 °C for 10 or 30 min, as described in the figure legends. Samples were centrifuged at 16,060 \(\times\) g for 1 or 2 min at 2 °C. Pellets and supernatants were analyzed by SDS-PAGE and fluoroxygraphy. Radiolabeled proteins were quantified by scintillation counting of bands extracted from dried gels as previously described (16).

**Digitonin Solubilization and Blue Native PAGE**—Thylakoid membranes were resuspended with one volume of 0.5 × IB, 20% glycerol, 0.5 M aminocaproic acid and one volume of 0.5 × IB, 20% glycerol, 0.5 M aminocaproic acid, 2% digitonin and incubated for 40 min at 0 °C, and the soluble fraction was collected after centrifugation at 100,000 \(\times\) g for 20 min. Samples were mixed with blue native sample buffer and subjected to electrophoresis on a 5–13.5% polyacrylamide gradient gel (8) and analyzed by fluoroxygraphy.

**Nondenaturing Coimmunoprecipitation**—40 μl of digitonin-solubilized thylakoid membranes from a precursor binding reaction were transferred to a low retention microcentrifuge tube containing \(\alpha\)Hcf106 IgG beads (equivalent to 40 μl of a 25% slurry solution).
and incubated for 60 min with end-over-end mixing at 4 °C. Pellets were recovered by centrifugation; washed twice with 200 µl of IB, 20% glycerol, 0.5 m aminoacapric acid, 0.5% digitonin; and transferred into Wizard micolumns (Promega). Elution was performed by incubating beads with 40 µl of 8 M urea, 5% SDS, 125 mM Tris-HCl, pH 6.8, for 90 min at 37 °C. The eluate (bound fraction) and unbound supernatant of the initial incubation were diluted with SDS sample buffer and analyzed by SDS-PAGE and fluorography.

**Factor Xa Assays**—Factor Xa was purchased from Novagen and buffer-exchanged into IB by desalting on 1-ml Sephadex G-25 spin columns. Proteolysis was initiated by the addition of factor Xa and terminated by either the addition of a 20 µM concentration of the specific factor Xa inhibitor, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone, dihydrochloride (Calbiochem), or addition of factor Xa cleavage of Escherichia coli produced TOE17 (University of Florida Protein Chemistry Core Facility, Interdisciplinary Center for Biotechnology Research).

**Protein Precipitation**—Proteins were precipitated by two different methods, as noted in the figure legends. First, samples received 10% trichloroacetic acid and were incubated for 45 min at 0 °C, centrifuged at 16,060 × g for 15 min, and washed with ice-cold acetone. Protein pellets were dried at the bench for 15 min and dissolved in sample buffer. Second, the samples received 90% ice-cold acetone and were incubated for 60 min at 0 °C, centrifuged at 16,060 × g for 15 min, dried at the bench for 15 min, and resuspended in sample buffer.

**Miscellaneous**—αTha4-treated thylakoids were prepared as described in Ref. 19 and resuspended at 1 mg of chlorophyll/ml in IB. Immunoblots were conducted using horseradish peroxidase-conjugated anti-rabbit Fc as secondary antibodies and ECL procedure (Amersham Biosciences). Methyl viologen, nigericin, and valinomycin were purchased from Sigma; KCl and urea were from Fisher.

**RESULTS**

A Position-specific Phenylalanine Substitution in the Signal Peptide Results in TOE17 That Forms a Tighter and More Stable Association with the cpTat Receptor Complex—As reported previously (13) and designated in Fig. 1A, (Tmd)Phe substitutions of certain residues in the TOE17 signal peptide resulted in a more stable association of the precursor with the 700-kDa receptor complex, cpTatC-Hcf106. One possibility for this effect was that the phenylalanine moiety of (Tmd)Phe increased the overall hydrophobicity of the signal peptide. In order to examine this possibility, phenylalanine substitutions were made at Val20 (V→20F), Ile19 (I→19F, Leu7 (L→7F), and Val3 (V3F) (Fig. 1A), and the effects on binding to the receptor complex were examined. All of the resulting precursors bound productively to thylakoids, since more than 90% of bound precursors were transported when submitted to a chase reaction (data not shown). Three of four modified precursors were similar to TOE17 with regard to their release from the membranes by salt washes and their behavior on BN-PAGE (Fig. 1, B and C). Thylakoid-bound TOE17 was largely extracted by 0.5 M KCl, as were I→19F, L→7F, and V3F (Fig. 1B). By contrast, bound V→20F, like its V→20F(Phe) counterpart, was not extracted by 0.5 M KCl but required a combination of KCl and urea to be dissociated from the membranes (Fig. 1B).

Similarly, V→20F-associated more stably with the 700-kDa receptor complex during BN-PAGE analysis than did TOE17 or the other substituted precursors (Fig. 1C, compare lane 2 with lanes 1 and 3–5). The different precursors showed some variability in the efficiencies of digitonin solubilization (see numbers below the gels in the top of Fig. 1C). However, the striking difference seen in Fig. 1C was due to the percentage of solubilized precursor that co-migrated with the 700-kDa receptor complex. For example, only 7.4% of solubilized TOE17 migrated at 700 kDa. This was previously attributed to a dissociation of bound TOE17 during electrophoresis, as evidenced by a smear of TOE17 below the precursor-receptor complex (Fig. 1C, lane...
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I) (8). By contrast, 75% of the solubilized V − 20F precursor co-migrated with the 700-kDa receptor complex.

From this set of data, we conclude that there are two types of productive interactions between the precursor and the thylakoid membranes. The first, exhibited by bound tOE17, is mediated by electrostatic interactions, which are relatively unstable to native electrophoresis. The second, exhibited by bound V − 20F, also involves conformational changes that render the precursor-receptor complex highly stable to native electrophoresis. The fact that L − 7F and V3F did not exhibit stronger binding comparable with their Tmd(Phe) counterparts (top of Fig. 1A) argues against the idea that stronger binding results from a general increase in hydrophobicity. Rather, it points to a position-specific effect of the phenylalanine, which notably, for V − 20F, is in the same relative location, with respect to the RR, as the consensus phenylalanine of the bacterial twin arginine motif.

KCl-extractable and KCl-resistant tOE17 Subpopulations Are Associated with the cpTatC-Hcf106 Receptor Complex—In light of the fact that a small subset of bound tOE17 was not extracted by 0.5 M KCl (Fig. 1B), we asked whether or not both the KCl-extractable and the KCl-resistant subpopulations of tOE17 were specifically associated with the cpTatC-Hcf106 receptor complex. BN-PAGE and co-immunoprecipitation analyses were conducted on precursor-bound membranes that were treated either with buffer or with 0.5 M KCl before digitonin solubilization (Fig. 2). tOE17 from both buffer-washed and KCl-washed membranes migrated at the location of the 700-kDa receptor complex. However, the amount of tOE17 at 700 kDa was substantially reduced by KCl treatment (Fig. 2A, lanes 2 and 3; see relative amounts below the lanes). KCl treatment affected neither the solubilization efficiency (Fig. 2B, lanes 4 and 8; see relative recovery numbers below the lanes) nor the BN-PAGE assay, as evidenced by the fact that the amount of V − 20F co-migrating with the 700-kDa receptor complex after KCl treatment was comparable with the amount from the digitonin-washed membranes (Fig. 2A, lanes 5 and 6). This was the first indication that the KCl-extractable and KCl-resistant subpopulations of thylakoid-bound tOE17 are associated with cpTatC-Hcf106.

Co-immunoprecipitation analysis confirmed these results (Fig. 2B). Specifically, 65% of the digitonin-solubilized tOE17 from buffer-washed membranes was co-immunoprecipitated with αHcf106 IgG beads (Fig. 2B, compare lanes 4 – 6). As expected, the KCl treatment reduced the amount of membrane-associated tOE17 to about 17% of the buffer-washed membranes (Fig. 2B, top, compare lanes 3 and 7), but most of the tOE17 solubilized from the KCl-treated membranes was co-immunoprecipitated with Hcf106 (Fig. 2B, top, lanes 8 – 10). Immunoblot analysis showed that virtually all of the cpTatC was co-immunoprecipitated by the beads and verified the specificity of immunoprecipitation, because chloroplast SecY was not co-immunoprecipitated (Fig. S1). Because essentially the same amount of bound V − 20F was solubilized and co-immunoprecipitated with or without the KCl wash (Fig. 2B, lanes 3 – 10), we conclude that the KCl treatment did not affect the co-immunoprecipitation assay.

Although these results show that both subpopulations of thylakoid-bound precursors are associated with cpTatC-Hcf106, some of the bound precursor was not accounted for in this analysis, because a significant amount of precursor was not solubilized by digitonin (Fig. 2B, compare numbers below lanes 3 and 4 and lanes 7 and 8). This could simply reflect inefficiency of the solubilization method. Alternatively, it could mean that some of the precursor is associated with the membrane bilayer, as has recently been reported for another precursor (20). To address this possibility, thylakoid membranes were pretreated with the protease thermolysin prior to a binding assay. This treatment, which digested all of the stromally exposed domains of cpTatC, Hcf106, and Tha4 (not shown), eliminated virtually all binding of tOE17 and V − 20F to the membranes (Fig. S2). This indicates that the observed precursor binding results from protein-protein interactions, which is consistent with our previous observations (11). It is likely that nearly all of the thylakoid-bound precursors are specifically associated with Tat protein components, because mutation of the twin arginine of...
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**tOE17 or V − 20Tmd(Phe)** to twin lysine virtually eliminates their ability to bind thylakoids (13).

**Binding Properties of tOE17 and V − 20F Are Determined by the Signal Peptide**—The dramatic effect of a single substitution of phenylalanine for valine in the signal peptide (Figs. 1 and 2) prompted us to assess whether the signal peptide, devoid of the mature domain, determines the nature of interactions between the precursor and the receptor complex. This was addressed by preparing transcription/translation constructs for the signal peptides of tOE17 and V − 20F. However, in order to resolve these peptides on Tris/Tricine gels, we found it necessary to include some amino-terminal residues of mature OE17. Constructs that contained the signal peptide and 17 or 30 mature domain residues were prepared from tOE17 (Sp17 and Sp30, respectively) and from V − 20F (SpF17 and SpF30, respectively).

All signal peptides efficiently bound to thylakoids (Fig. 3A, even lanes). When signal peptide bound thylakoids were subjected to BN-PAGE analysis, the signal peptides exhibited the behavior of the precursors from which they were derived. Strong bands of SpF17 and SpF30 signal peptides co-migrated with the 700-kDa receptor complex (Fig. 3B, lanes 5 and 6). Although not visible in Fig. 3B (lanes 3 and 4), Sp17 and Sp30 gave faint bands co-migrating with the 700-kDa receptor complex with a longer fluorescent exposure (data not shown). The four signal peptides also exhibited similar extraction behavior as their parent precursors, namely extractable with KCl for Sp17 and Sp30 and resistant to KCl (but extractable by a combination of KCl and urea) for SpF17 and SpF30 (Fig. 3C). These results strongly suggest that the signal peptide alone determines the nature of binding to the cpTatC-Hcf106 receptor complex.

**Signal Peptide-flanking Regions of tOE17, but Not V − 20F, Are Accessible to Factor Xa Protease from the cis Side of the Membrane**—The fact that KCl and urea are necessary to dissociate bound V − 20F from thylakoid membranes (Fig. 1) suggested that the V − 20F signal peptide might be more deeply buried in the cpTatC-Hcf106 binding site. To assess this possibility, factor Xa cleavage sites (IEGR) were engineered into the amino- and carboxyl-proximal regions flanking the signal peptides, and the accessibility of bound precursors to factor Xa was determined. Unmodified tOE17 was cleaved by factor Xa at a cryptic site adjacent to the Tpp (thylakoid-processing peptidase) cleavage site (see “Experimental Procedures” and Fig. 4A). This site was used as the most carboxyl-proximal site to the signal peptide. When we compared the cryptic site accessibility of productively bound tOE17 and V − 20F, we observed a dramatic difference (Fig. 4B, lanes 4–7). The tOE17 cryptic site was largely accessible to factor Xa, as evidenced by the linear appearance of the cleavage product (Xcp) during the time of the assay. By contrast, the V − 20F cryptic site was completely protected from proteolysis, since no cleavage product could be detected. As controls, the translation products of both precursors were treated with factor Xa and found to be equally susceptible to cleavage (Fig. 4B, lane 8).

Two additional carboxyl-proximal sites were engineered at residues 11 (Fig. 5A) and 39 (Fig. 4C) of the mature OE17 domain. This region is reported by x-ray crystallography to be relatively unstructured, unresolved in one study and showing limited β structure in a second study (21, 22). Most surprisingly, and just like for the cryptic site, the two additional carboxyl-proximal sites of bound tOE17 were readily cleaved by factor Xa, whereas the same sites of V − 20F were essentially inaccessible (see Fig. 4D for IEGR11 and Fig. 5B for IEGR11).

In order to determine the accessibility of the amino-proximal side of the signal peptide, iOE17 and iOE17V − 20F were engineered to contain a factor Xa cleavage site at position −30 (Fig. 5A). iOE17 is the natural intermediate of OE17, which is transiently present in the stroma following import into the chloroplast and before transport across the thylakoid membrane. iOE17 contains 11 residues that are not essential for protein transport but appear to modulate precursor binding, since iOE17 binds less efficiently than tOE17, which lacks these 11 residues (11). The IEGR-engineered iOE17 and iOE17V − 20F, which interestingly bound to thylakoids more efficiently than iOE17 (data not shown), exhibited the same characteristics of productive binding as their tOE17 and tOE17V − 20F counterparts (as assessed by salt and urea extractability and BN-PAGE analysis; data not shown). Again, a similar difference in accessibility was observed for the two precursors, namely the amino terminus of the bound iOE17 was very rapidly and completely accessible to factor Xa cleavage, whereas the amino terminus of iOE17V − 20F was essentially inaccessible to factor Xa (Fig. 5B, lanes 4–7).

**Domains from cpTatC and Hcf106, rather than the Mature Domain, Are Likely to Mask the V − 20F Signal Peptide from Factor Xa Digestion**—The above results indicate that thylakoid-bound tOE17 (iOE17) is associated peripherally with the
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FIGURE 4. The signal peptide carboxyl-flanking region of thylakoid-bound tOE17 is highly accessible to factor Xa, whereas the same region of bound V − 20F is not accessible. A, iOE17 signal peptide sequence is shown, with the positions of the cryptic factor Xa and the Tpp cleavage sites above the sequence. The underlined residues are those determined by NH₂-terminal sequencing of E. coli-produced TOE17 digested by factor Xa. The twin arginine consensus is represented in boldface type, and the phenylalnine substitution for valine at position −20 (V − 20F) is indicated below the sequence. B, tOE17 and V − 20F were incubated with pea thylakoids in a binding reaction (b) and divided into two samples (1 mg of chlorophyll/ml). One sample was incubated under transport conditions in a chase reaction (c). The second sample was treated with 3 units of factor Xa per 20 µg of chlorophyll at 0 °C, in darkness. Aliquots were taken after 2.5, 5, 7.5, and 10 min and immediately quenched with SDS sample buffer. A sample of translation product was treated with 3 units/20-µl reaction at 0 °C for 10 min. C, sequence of the amino-terminal OE17 mature domain with the IEGR₉₉ underlined and the factor Xa cleavage site indicated above the sequence. The amino acid insertions to engineer the cleavage site are in italic type. In addition, arginine at position −2 (see A) was replaced with aspartate to remove the cryptic and either incubated in a chase reaction or treated with factor Xa as described above in B. tp, in vitro translated precursor equivalent to 2.5% (lane 1) and 12.5% (lane 8) of that used for binding reactions; p, precursor form; Xcp, factor Xa cleavage product; asterisk, mature OE17 obtained upon transport to the lumen. Panels are fluorograms of SDS-PAGE.

cpTatC-Hcf106 receptor complex, whereas V − 20F (iOE17V − 20F) is bound in such a way that the signal peptide and flanking regions are masked from factor Xa. Although it is likely that proteins provide this masking, it was conceivable that the V − 20F signal peptide and flanking regions are embedded in the lipid bilayer. In order to address this possibility, tOE17 and V − 20F were bound to thylakoids, and the recovered thylakoids were treated with the broad specificity protease thermolysin. As seen in Fig. 6A, the precursors were completely degraded, with no radioactivity evident at the relative location of the signal peptide (lanes 4 and 8). This indicates that the V − 20F signal peptide is masked from factor Xa by protein rather than lipids. The most likely possibilities are either that cpTatC-Hcf106 masks the cleavage site or that a tightly folded OE17 mature domain acts like a lid on the bound signal peptide and thereby prevents access. In order to distinguish these scenarios, Sp₃₀ and SpF₃₀ signal peptides, lacking the mature domain, were bound to thylakoids, which were then subjected to either factor Xa or to thermolysin treatments. Factor Xa post-treatment revealed that only Sp₃₀ was susceptible to cleavage at its cryptic site (Fig. 6B, lane 6), whereas SpF₃₀ remained completely inaccessible (lane 12). This indicates that the OE17 mature domain is not responsible for masking the signal peptide at the carboxyl-proximal cryptic site. Although it is still a formal possibility that cleavage sites at 11 and 39 residues into the mature domain are differentially protected in V − 20F as opposed to tOE17 because of tight folding, that seems unlikely, because the two precursors have identical mature domains and because the factor Xa sites were introduced into the least structured region of mOE17, as mentioned above. As with the full-size precursors, both the bound Sp₃₀ and bound SpF₃₀ were completely degraded by thermolysin (Fig. 6B, lanes 4 and 10). These results strongly suggest that V − 20F signal peptide is bound deep within the cpTatC-Hcf106 receptor complex and that domains of cpTatC and possibly Hcf106 prevent access to factor Xa. This is in agreement with our previous study showing that the tightly bound Tmd(Phe) derivatives of tOE17 cross-linked solely to cpTatC and Hcf106 (13).

Bound tOE17 Is More Tightly Associated with Thylakoids That Are Energized with a Proton Gradient—During a normal transport reaction, the thylakoid membranes are energized with a proton gradient, a Tha₄ oligomer assembles, and the protein is transported. However, because precursors are only transiently associated with the translocase before being translocated, we devised an assay to specifically assess if precursor-receptor interactions are different when thylakoids are energized with a proton gradient. This was accomplished by a previously characterized method of pretreating thylakoids with
The signal peptides derived from V − 20F are buried in a binding pocket likely involving domains of cpTatC and Hcf106. A, in vitro translated tOE17 and V − 20F precursors were incubated with thylakoids in a binding reaction (b) as described under "Experimental Procedures" and shown above the panels. Thylakoids were recovered and resuspended in IB at 0.5 mg of Chl/ml and divided into two 50-μl aliquots. One aliquot received thermolysin (0.33 mg/ml) and was incubated for 45 min at 0 °C. Both aliquots were given 75 μl of IB, 14 mM EDTA, and the thylakoids were recovered by centrifugation, washed with 100 μl of IB, 5 mM EDTA, resuspended in sample buffer, and analyzed by 16% Tris/Tricine SDS-PAGE and fluorography. TP, translation product equivalent to 2.5% of that used for binding reactions. Sp30 translation product was loaded in lane 9 as a size marker. B, in vitro translated Sp30 and SpF30 signal peptides were incubated with thylakoids in a binding reaction (b). Thylakoids were recovered and resuspended in IB and divided into two 50-μl aliquots (0.5 mg of chlorophyll/ml) and two 25-μl aliquots (1 mg of chlorophyll/ml). The two 50-μl aliquots were treated with thermolysin as in A. The two 25-μl aliquots received either buffer or 1 unit of factor Xa and were incubated for 15 min at 0 °C, and the reaction was stopped with 20 μl of 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone, dihydrochloride, the factor Xa inhibitor, Sp30, and SpF30 samples were precipitated with 90% acetone, resuspended in sample buffer, and analyzed by 16% Tris/Tricine SDS-PAGE and fluorography. TP, translation product equivalent to 5% of that used for binding reactions. The precursors (p) and the factor Xa cleavage product (XCP) are indicated by the arrows.

αTha4 IgGs, which prevents translocation without impairing precursor binding or the ability to generate a proton gradient (19). TOE17 was bound to αTha4-treated membranes, washed to remove unbound TOE17, and incubated for 5 min in the light to generate a proton gradient. The TOE17-bound membranes were then treated with buffer or increasing concentrations of buffered KCl solutions, incubated for an additional 10 min in the light, and then rapidly centrifuged to separate membranes from supernatant (Fig. 7A). For comparison, TOE17-bound αTha4-treated thylakoids were similarly treated in the absence of a proton gradient, which was achieved by incubation either in darkness (Fig. 7) or in the light but with ionophores to dissipate the proton gradient. Thylakoid-bound TOE17 became twice as resistant to salt extraction in the presence of a proton gradient as compared with thylakloid-bound TOE17 without a proton gradient (Fig. 7, A (compare lanes 8–10 with lanes 4–6) and B). Essentially the same results were obtained in several experiments that compared light-incubated membranes with light plus ionophores membranes (data not shown). Interestingly, when light-incubated membranes were returned to darkness (no proton gradient), the bound TOE17 regained its property of being extractable by KCl solutions, indicating that the proton gradient effect is transient and reversible (Fig. 7C). These results strongly support the idea that TOE17 precursor binds more tightly to thylakoid membranes when a proton gradient is available.

As a second test for the nature of TOE17-receptor interactions under energized conditions, accessibility to factor Xa of the cpTatC nucleotide binding pocket likely involving domains of cpTatC and Hcf106. A, in vitro translated tOE17 was incubated with αTha4-treated pea thylakoids in a binding reaction (b). Recovered thylakoids were resuspended at 0.33 mg of chlorophyll/ml with two-thirds volume of IB, one-third volume of HK, 10 mM MgCl2, and 50 μM methyl viologen, and divided into 12 aliquots of 75 μl. Eight aliquots were incubated for 5 min in a water bath at 15 °C in 70 μM/l/m/s’ white light to generate a proton gradient (ΔpH), and four aliquots were incubated in darkness (No ΔpH). A, four samples incubated in light and the four samples incubated in darkness received 10 μl of buffer (HK), 1, 2, or 4 × buffered KCl, respectively, for final concentrations of 0, 0.125, 0.25, and 0.5 M KCl, and were incubated for an additional 10 min under the same conditions (light or darkness). Assays were immediately centrifuged at 16,060 × g for 1 min in a microcentrifuge to separate the thylakoids (pellets) from the supernatants. The panels represent SDS-PAGE fluorograms of the samples. TP represents 2.5% of that used for the binding assay. B, a bar chart of the average and S.E. from five independent experiments. White bars, proton gradient conditions (light; ΔpH); black bars, no proton gradient conditions (darkness; No ΔpH). C, the additional four aliquots incubated in light in A were pelleted for 4 min at 3,300 × g at 2 °C. Membranes were resuspended in 85 μl of buffer or buffered 0.125, 0.25, 0.5 M KCl and incubated in a water bath at 15 °C for 10 min in darkness (No ΔpH). Membranes (pellets) and supernatants were separated by centrifugation at 16,060 × g for 1 min in a microcentrifuge. Fluorograms from SDS-PAGE are shown.
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**A.**

| Lanes | \( \Delta p \)H | No \( \Delta p \)H | Time (min) |
|---|---|---|---|
| 1 | 2.5 | 5 | 10 |
| 2 | 2.5 | 5 | 10 |
| 3 | 2.5 | 5 | 10 |
| 4 | 2.5 | 5 | 10 |
| 5 | 2.5 | 5 | 10 |
| 6 | 2.5 | 5 | 10 |
| 7 | 2.5 | 5 | 10 |
| 8 | 2.5 | 5 | 10 |
| 9 | 2.5 | 5 | 10 |
| 10 | 2.5 | 5 | 10 |

**B.**

| Lanes | \( \Delta p \)H | No \( \Delta p \)H | Time (min) |
|---|---|---|---|
| 1 | 2.5 | 5 | 10 |
| 2 | 2.5 | 5 | 10 |
| 3 | 2.5 | 5 | 10 |
| 4 | 2.5 | 5 | 10 |
| 5 | 2.5 | 5 | 10 |
| 6 | 2.5 | 5 | 10 |
| 7 | 2.5 | 5 | 10 |
| 8 | 2.5 | 5 | 10 |
| 9 | 2.5 | 5 | 10 |
| 10 | 2.5 | 5 | 10 |

**FIGURE 8.** The regions flanking the signal peptide of bound tOE17 become less accessible to factor Xa in the presence of a proton gradient. aTha4-treated pea thylakoids were incubated with in vitro translated tOE17 or iOE17 IEGR \(-30\), in a binding reaction (b). Recovered thylakoids (1 mg of chlorophyll/ml in IB buffer, 10 \( \mu \)M methyl viologen) were divided into two samples and incubated for 5 min at 15 \( ^\circ \)C in the light to generate a proton gradient (\( \Delta p \)H) or in darkness (No \( \Delta p \)H). Samples received factor Xa, and aliquots were taken after 2.5, 5, 7.5, and 10 min, immediately quenched with SDS sample buffer, tp, translation product equivalent to 2.5% of that used for binding reactions. A, accessibility of the carboxyl-terminal side of tOE17 signal peptide was assessed by factor Xa cleavage of its cryptic site (1.5 units/20 \( \mu \)g of chlorophyll). B, accessibility of the amino-terminal side of the iOE17 signal peptide was assessed by factor Xa cleavage of the IEGR \(-30\) (0.05 units/20 \( \mu \)g of chlorophyll). A and B, fluorograms of SDS-polyacrylamide gels. The panels in A are from the same gel and film but are separated for visual effect. Similarly, the panels in B are from the same gel and film. The arrows indicate the precursor (p) and the factor Xa cleavage product (Xcp). The numbers under each panel represent the percentage of bound precursor cleaved by factor Xa.

Cryptic site, evidenced by a decrease in the rate of cleavage of bound tOE17, was reproducibly observed in the presence of the proton gradient (Fig. 8A, lanes 3–6), compared either with dark-incubated thylakoids (lanes 7–10) or with those incubated in the light with ionophores (data not shown). Similarly, the amino-proximal site in the iOE17 signal peptide was less accessible in the presence (Fig. 8B, lanes 3–6) than in the absence (lanes 7–10) of a proton gradient, as visualized by the differences in the amount of uncleaved precursors in each condition. These results suggest that bound tOE17 signal peptide becomes more deeply buried in the receptor complex when the membrane is energized by a proton gradient.

**DISCUSSION**

In plants, substrates for the thylakoid Tat system are translated in the cytosol with amino-terminal bipartite targeting sequences. The amino-proximal targeting domain directs import of the precursors into the chloroplast and is removed in the stroma by the stromal processing protease, generating intermediate precursors with exposed signal peptides. Several studies have investigated initial interactions of Tat pathway precursor proteins with the thylakoid membrane and Tat components (9, 11, 15, 23, 24). None of these studies used physiological precursors, because the physiological substrates bind poorly to thylakoids. For example, of the intermediate precursors examined, iOE23 and iPS1N do not stably bind thylakoids without a proton gradient, and iOE17 exhibits only moderate binding (11). However, certain modifications of precursors, a deletion of 11 amino-terminal iOE17 residues to generate tOE17 (11, 15), or a substitution of 2 of the 11 residues in iOE17 to generate iOE17 IEGR \(-30\) (Fig. 5), result in an irreversible binding to the receptor complex in the absence of a proton gradient. Similarly, substituting the H- and C-domains with the analogous domains of a Sec pathway signal peptide results in precursors that associate very stably with the receptor complex (15).

Until the present study, it was a mystery why natural precursors bind poorly and modified precursors bind tightly, although both types of precursors are efficiently transported. To address this question, we examined the binding characteristics of tOE17 and a slightly modified form of tOE17 that binds very tightly to the cpTatC-Hcf106 receptor complex (V \( \rightarrow \) 20F). In addition, we devised an experimental method to examine binding that occurs in the presence of a proton gradient. Our results indicate that in energized conditions, precursors bind tightly to the receptor complex and become more intimately associated with cpTatC and Hcf106. They further indicate that certain minor changes in the makeup of the signal peptide allows it to better “fit” the binding pocket in the absence of a proton gradient.

Here we have characterized two types of affinity between precursor and the receptor complex. tOE17 binds via electrostatic interactions, forms a semiseminal complex with cpTatC-Hcf106, and is exposed peripherally on the surface of the receptor complex as determined by accessibility to factor Xa. The fact that the tOE17 signal peptide alone displays the same binding characteristics as the full precursor suggests that tOE17 is held to the receptor complex via its twin arginine and the corresponding acidic residues that likely reside on cpTatC (25). V \( \rightarrow \) 20F binds with a higher affinity that requires salt and urea to disrupt and forms a very stable complex with cpTatC-Hcf106. The requirement for urea suggests that some protein unfolding is needed for signal peptide release. The differential effects of factor Xa and thermolysin on the bound signal peptide strongly suggest that it is buried more deeply within the proteinaceous cpTatC-Hcf106 binding cavity and is not inserted into the lipid bilayer as has been suggested for a COOH-terminally modified and bacterially expressed pOE17 and also for a chimeric Tat pathway precursor (23, 24).

In the present work, a large number of site-specific mutations were made in or near the tOE17 (iOE17) signal peptide, including phenylalanine substitutions at four different locations in the H-domain, alterations of the N-domain, and substitutions in the Tpp cleavage site. Of these, only Val \( \rightarrow \) 20F resulted in significantly altered binding affinity to the receptor complex. The fact that a phenylalanine substitution only at Val \( \rightarrow \) 20 increased binding affinity to the receptor complex argues against a hydrophobic effect and suggests that the substituted phenylalanine serves as a structural determinant in the binding interaction. This is supported by the observation that (Tmd)Phe substitution at the same site (Val \( \rightarrow \) 20F) produced unquestionably the most abundant cross-linking product, to cpTatC (13). It is interesting that the phenylalanine in V \( \rightarrow \) 20F, two residues from the RR, corresponds to the phenylalanine in the consensus motif for bacterial Tat pathway substrates (S/T)RRXFLK. After the twin arginine, the phenylalanine is the
most highly conserved residue of bacterial Tat signal peptides (5), and its substitution by other amino acids dramatically impairs transport efficiency. For example, mutation of the *E. coli* PreSufI consensus phenylalanine to tyrosine or alanine severely retarded transport (26), and substitutions of the consensus phenylalanine of the *Streptomyces lividans* xylanase signal peptide reduced transport to 25% of wild type (27). A similar reduction was observed for lysine substitution of one of the two arginines (26, 27). Mutations of other consensus residues have far less dramatic effects (26). Previous studies have demonstrated that bacterial Tat signal peptides are very effective in directing transport by the thylakoid Tat pathway (28, 29), and the experiments presented here show the dramatic effect of the consensus phenylalanine on precursor binding to the cpTatC-Hcf106 complex. This makes it all the more puzzling that nearly all known or predicted substrates of the thylakoid Tat pathway lack the consensus phenylalanine.

Our experiments showing the increased stability of thylakoid-bound tOE17 in the presence of the proton gradient may offer an explanation for this seeming paradox. In the presence of the proton gradient, thylakoid-bound tOE17 acquired characteristics similar to those of thylakoid bound V − 20F in the absence of a proton gradient. This suggests that the proton gradient induces some conformational change or changes that allow signal peptides lacking the consensus phenylalanine to enter the more advanced binding site of the receptor. For example, the proton gradient might induce a conformational change in cpTatC-Hcf106, in the precursor, or in both to create a better fit between signal peptide and receptor. On the other hand, the better fitting twin arginine motif of a precursor like V − 20F might alternatively cause conformational changes of the precursor or might induce a conformational change in the receptor upon binding. Of interest in this regard is that signal peptide binding to energized thylakoids also triggers the conformational rearrangement and assembly of oligomeric Tha4 with cpTatC-Hcf106 to form the translocase (9, 10).

Although future mechanistic studies will be necessary to distinguish these possibilities, our basic observation regarding the effect of the proton gradient on binding characteristics explains the previously puzzling finding that 150 mM KCl, while inhibiting the binding of tOE17 in the absence of a proton gradient, did not significantly impair transport of tOE17 (12). Thus, precursors, such as iOE23, which do not bind stably in the absence of a proton gradient, may bind more tightly in the presence of a proton gradient, explaining their efficient transport by the Tat pathway. In fact, we have observed binding of iOE23 to energized, αTha4-treated thylakoids. Consistent with this interpretation is our observation that tOE17 and V − 20F exhibit virtually identical transport rates, despite the obvious differences in binding affinity in the absence of the proton gradient.  

This feature of the thylakoid Tat system may be a regulatory mechanism peculiar to plants. Whereas bacterial plasma membranes are energized throughout their life cycle, plant thylakoids are energized only during the day. During the nighttime, the lack of light and inactivation of the proton pumping activity of the ATP synthase leaves the thylakoid membrane without a significant proton gradient. A recent in vivo study of GFP transport by the thylakoid Tat system and fluorescence in the lumen nicely illustrates this point (30). GFP was transported to the lumen during the day but was not fluorescent due to the low luminal pH. During the night, luminal GFP was fluorescent, but GFP expressed during the night period accumulated in the stromal compartment. If our speculative interpretation is correct, it would indicate that during the nighttime, any accumulated Tat substrates would only reversibly interact with the Tat machinery, such that upon energization, all substrates would have access to the translocase based on their relative abundance. Since Tat-transported proteins are subunits of all of the major photosynthetic complexes, this may help to ensure coordinated assembly of the photosynthetic apparatus.

Regardless of the physiological reason for protonmotive force-dependent binding to the thylakoid Tat apparatus, our results here provide an experimental system to assess the signal peptide determinants that match the receptor binding site during active transport. Considering the absence of detailed structural information on the receptor binding site, this approach may lead to an increased understanding of the manner by which the Tat receptor complex interacts with its substrates and the molecular basis by which signal peptide binding triggers recruitment of Tha4 to form the translocase.

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