Precursors Bind to Specific Sites on Thylakoid Membranes prior 
To Transport on the Delta pH Protein Translocation System*

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The Delta pH pathway is one of two systems for protein transport to the thylakoid lumen. It is a novel translocation system that requires only the thylakoidal ΔpH to power translocation. Several substrates of the Delta pH pathway, including the intermediate precursor form of OE17 (tOE17) and the truncated precursor form of OE17 (tOE17), were shown to bind to the membrane in the absence of the ΔpH and be transported into the lumen when the ΔpH was restored. Binding occurred without energy or soluble factors, and efficient transport from the bound state (∼80–90%) required only the ΔpH. Binding is due to protein-protein interactions because protease pretreatment of thylakoids destroyed their binding capability. Precursors are bound to a specific site on the Delta pH pathway because binding was competed by saturating amounts of Delta pH pathway precursor proteins, but not by a Sec pathway precursor protein. These results suggested that precursor tOE17 binds to components of the Delta pH pathway translocation machinery. Hcf106 and Tha4 are two components of the Delta pH pathway machinery. Antibodies to Hcf106 or Tha4, when prebound to thylakoids, specifically inhibited precursor transport on the Delta pH pathway. However, only Hcf106 antibodies reduced the level of precursor binding. These results suggest that Hcf106 functions in early steps of the transport process.

In plants, the photochemical reactions leading to ATP and reducing power take place on the thylakoid membranes within the chloroplast. Thylakoids contain well over 100 polypeptides, most of which are nuclear-encoded and synthesized in the cytosol as precursor proteins with transient amino-terminal transit peptides (see Ref. 1 for review). For a thylakoid lumen protein, its transit peptide consists of two parts: 1) a stroma-targeting domain, and 2) a lumen-targeting domain. The stroma-targeting domain governs import of the precursor across the chloroplast envelope and is cleaved by a processing protease in the stroma, resulting in an intermediate precursor. The exposed lumen-targeting domain directs thylakoid translocation and is cleaved by a lumen-facing processing protease.

There appears to be one major pathway for protein import across the chloroplast envelope. However, there are two pathways for protein transport into the thylakoid lumen (1–3). One pathway is called the thylakoid Sec pathway because this pathway uses chloroplast homologues of bacterial SecA, SecY, and SecE proteins (4–7). A second pathway is called the Delta pH pathway because, for this pathway, the ΔpH across the thylakoid membrane is the only energy requirement for protein transport. Each pathway is specific for a subset of precursor proteins.

The Delta pH pathway was first recognized as a distinct transport pathway when it was found that certain precursors, specifically OE17 and OE23, uniquely require neither soluble protein factors nor NTPs for transport but only the thylakoidal ΔpH (8). A requirement for specific translocation machinery was demonstrated by precursor competition studies (9) and by the demonstration that protease treatment of thylakoids destroys their ability to transport Delta pH pathway substrates (10). Two thylakoid proteins, Hcf106 and Tha4, were recently identified as components involved in transport on the Delta pH pathway (6, 11, 12), and several components of a homologous system have been identified in bacteria (13–15). However, neither the mechanism of Delta pH pathway transport nor the function of its components is known with any certainty. One limitation has been that intermediate steps of the process have not been identified.

In this study, we have identified a bound intermediate on the pathway. Certain truncated Delta pH pathway precursors bind tightly to the thylakoid membrane in the absence of the ΔpH and were transported into the lumen when the ΔpH was restored. The characteristics of precursor binding and the subsequent chase of bound precursor suggest that the precursor is binding to a component of the transport machinery. Antibodies to Hcf106 reduced the extent of binding, whereas antibodies to Tha4 had no effect on binding. These results suggest that precursor binding represents the first committed step of the Delta pH pathway.

**EXPERIMENTAL PROCEDURES**

Materials—All reagents and enzymes were from commercial sources. In vitro transcription plasmids for OE23 (9), OE23, tOE17, iOE17, intermediate precursor form of photosystem I subunit N, DT17, and DT23 (16) have been described. Capped RNA was produced with SP6 polymerase (Promega) and translated in a wheat germ system (17) in the presence of [3H]leucine.

Overexpression of tOE17, DT23, and pOE33 in Escherichia coli—tOE17 was expressed as a glutathione S-transferase fusion protein in E. coli. The coding sequence of tOE17 was amplified with the forward primer 5’-CGTTGCGGATCCGCGCCCGCCCGTCGTGATCG-3’ and reverse primer 5’-CCATTATAGATTACGCGGTCTAGCTTGG-3’

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1 The abbreviations used are: OE17, OE33, and OE23, the 17-, 33-, and 23-kDa components of the oxygen evolving complex; pOE33/OE23/OE17, OE33/OE23/OE17, precursor, intermediate precursor, and truncated precursor forms of OE33/OE23/OE17; CCCP, carbonyl cyanide m-chlorophenylhydrazone; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

10016 This paper is available on line at http://www.jbc.org
chloroplasts were isolated from 9–10-day pea seedlings (Laxton’s Pro-
colI acids MA, residues. Whereas in vitro tOE17 was released from glutathione
derived from the thylakoid membrane when protein transport is conducted essentially as de-
scribed previously (9). Precursor binding and chase assays were con-
ducted by incubating 10 μl of 3H-labeled in vitro translated precursor with thylakoids (25 μg of chlorophyll) in the presence of 5 μM CTP or
2 units of apyrase for 15 min at 0 °C in a total volume of 75 μl of import
buffer. Thylakoid membranes were recovered by centrifugation at 3,200 × g, washed twice with import buffer, and divided into two equal portions in new microcentrifuge tubes. One portion was directly ana-
yzed to assess the amount of bound precursor. The second portion was resuspended in 75.5 μl of import buffer containing ~200 μg of protein of
stromal extract and 1 μM dithiothreitol and assayed for chase of bound precursor by transfer to a 25 °C illuminated water bath for 15 min. Unless noted otherwise, thylakoids recovered from transport and chase assays were post-treated with thermolysin (18).

Preparation of Thermolysin- or Protease K-treated Thylakoids for Use in Binding and Transport Assays—Thermolysin-treated thylakoids were prepared by incubating washed thylakoids at 0.5 μg chlorophyll/μl import buffer with 0–160 μg thermolysin/μl (from a 2 mg/ml stock containing 10 mM CaCl₂) for 60 min at 0 °C. The thylakoid membranes were then recovered by centrifugation, washed twice with import buffer, and either analyzed directly (top panel) or treated with protease (bottom panel) and analyzed by SDS-PAGE and fluorescence. Each lane contains thylakoid membranes equivalent to 50% of that present in each assay. Lane tp contains 5% of the precursor added to the assay. The positions of the precursor iOE17 (p) and the mature form (m) are marked. B. THALET precursor was incubated with thylakoids in the dark plus apyrase for 15 min on ice. The thylakoids were then recovered by centrifugation through a 10% Percoll cushion, resus-
pended in 150 μl of import buffer, and divided into six aliquots. One aliquot was analyzed directly (P); the remaining aliquots were washed one to five times with 0.5 ml of import buffer transferred to new microcentrifuge tubes, and analyzed by SDS-PAGE fluorescence, fol-
lowed by scintillation counting of radiolabeled proteins extracted from gel bands (18).

**RESULTS**

**Delta pH Pathway Precursors Bind to the Membrane in the Absence of a ΔpH**—Protein transport by the Delta pH pathway is presumed to start with a precursor recognition event on the thylakoid membrane. However, stable precursor binding to thylakoids had not been observed previously. We surveyed a number of precursors, both naturally occurring and recombinant precursors, and found several that bound to the thylakoid membrane in the absence of ΔpH (see below). For this study, it was important to use precursors that lacked the stroma-targeting domain of the transit peptide to avoid binding between precursors and lipids or components of the envelope import system (19, 20).

**tOE17 is an amino-terminally truncated form of iOE17 that we constructed as part of another study on targeting determinants** (16). It lacks an acidic amino-proximal extension that is not essential for transport into isolated thylakoids (16). Previous work (16) showed that tOE17 is an extremely efficient substrate, exhibiting at least two times the initial rate of transport compared to the full-length precursor pOE17, or the authentic stromal intermediate, iOE17. Fig. 1A shows the results of transport assays with tOE17 under varying conditions. As with other Delta pH pathway substrates, tOE17 transport was independent of stromal extract (lane 5), unaffected by azide (lane 6), an inhibitor of SecA, and independent of ATP (lane 4),
provided that a ΔpH was generated by illuminating the assays. On the other hand, tOE17 transport was eliminated by dissipating the thylakoidal ΔpH with ionophores (lane 3) or by carrying out the incubation in the dark with apyrase to deplete ATP (lane 1), which prevents ATP from powering the ATP synthase to generate a pH gradient (8). When tOE17 transport was inhibited, a substantial quantity of precursor remained bound to the thylakoid membrane. Bound precursor was degraded by protease treatment of thylakoids, demonstrating that it was exposed on the exterior of the thylakoids. Other experiments showed that binding occurred equally well in the presence or absence of stromal extract (data not shown).

Sequential washing of tOE17-bound thylakoids assessed the strength and nature of precursor binding. As can be seen in Fig. 1B, a considerable percentage of added tOE17 co-sedimented with thylakoids through a Percoll cushion (lane P). After two washes with import buffer, the amount that remained associated with the membrane became relatively constant. For most of the studies reported here, membranes were washed twice following precursor binding prior to subsequent analysis. Although tOE17 appears tightly bound to the membrane, it is not buried in the bilayer because it was entirely removed by 0.1 M sodium carbonate extraction and largely removed by a 6 M urea wash (data not shown).

Bound Precursor Proceeds into the Lumen upon Restoration of ΔpH—If tOE17 binding is physiologically relevant, then bound precursor should proceed into the thylakoid lumen upon restoration of the ΔpH. We examined several ways to conduct binding assays that would allow re-establishment of the thylakoidal pH gradient. Initial attempts to simply carry out incubations on ice, and nigericin and valinomycin were added to all assay mixtures at a final concentration of 0.5 and 1 μM, respectively. Thylakoid membranes were recovered by centrifugation, washed twice with import buffer, and analyzed directly (lanes 1 and 3) or treated with protease (lanes 2 and 4) and then analyzed by SDS-PAGE/fluorography. Each lane contains the 50% of the thylakoid membranes present in each assay. Lane tp contains 5% of the precursor added to each assay. Precursors used are shown to the left of each panel.

The ability of bound precursor to be transported into the lumen was tested by incubating tOE17-bound thylakoids at 25 °C in the light. About 80% of bound precursor was transported into the lumen (Fig. 3A), as judged by its being processed to mature size (lanes 4 and 5) and becoming protected from exogenous protease (lane 6). The time required for chase of about 50% of the bound precursor was 2.5 min, comparable to the half-time for transport of newly added precursor (data not shown). These results indicate that the bound tOE17 is a productive intermediate on the Delta pH pathway.

Other precursors that can be transported on the Delta pH pathway, most of them constructed in our laboratory, were also tested in binding and chase assays. As shown in Fig. 3B, the ability to bind tightly to the thylakoid membrane varied among these precursors. tOE17, DT17, and DT23 bound strongly to the thylakoid membrane, and a substantial percentage of

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\[^2\]X. Ma and K. Cline, manuscript in preparation.
bound precursor was chased into thylakoid lumen. iOE17 exhibited moderate binding, and some bound precursor was chased into thylakoids. Intermediate precursor form of photosystem I subunit N exhibited moderate binding, but none of the bound intermediate precursor form of photosystem 1 subunit N was productive. There was hardly any binding for the stromal intermediate iOE23 or for a truncated version, tOE23. The reason that some precursors can productively bind better than others is not clear. However, it is of interest that binding ability correlates with the relative ability of precursors to be transported at 0 °C (Figs. 2 and 3).

Transport of Bound Precursors Requires Only the Thylakoid \( \Delta pH \)—The requirements for transport of bound precursor were determined by incubating \( \text{tOE17} \)-bound thylakoids under different conditions (Fig. 4). Chase was completely inhibited by dissipating the \( \Delta pH \) by ionophores in the light (lane 7) or by preventing its formation by dark plus apyrase (lane 8). Apyrase treatment in the light did not inhibit the chase (lane 9). These results indicate that \( \Delta pH \) is required for transport of bound precursor. Substantial chase occurred in buffer plus light alone (lane 2) and was enhanced by including MgCl\(_2\) (lane 3). Stromal extract was not required but sometimes had a stimulatory effect (lanes 5 and 6). As expected, ATP had little effect on chase efficiency (lane 4). We also examined the chase requirements for DT23 and iOE17 and found them to be identical to those for tOE17 (data not shown), i.e., transport from the bound state requires only the thylakoidal pH gradient.

\textbf{tOE17 Binding Is Due to Protein-Protein Interactions}—To determine whether thylakoid proteins mediate precursor binding, thylakoids were pretreated with protease (Fig. 5). Precursor binding to the protease-treated thylakoids was substantially reduced compared with buffer-washed thylakoids (Fig. 5A). Proteinase K pretreatment had less of an effect on precursor binding than thermolysin treatment. The subsequent chase of any bound precursor from either thermolysin- or proteinase K-treated thylakoids was totally inhibited (Fig. 5B), indicating that protease treatment damaged the transport machinery and/or the ability of thylakoids to generate a pH gradient. This was also demonstrated by assaying the treated membranes for transport of fresh precursor (Fig. 5C). Analysis of entire binding assay mixtures verified that no traces of protease remained after the washing steps because no degradation of precursor occurred during the assay (Fig. 5D). These data indicate that surface-exposed proteins of the thylakoid membrane are involved in the binding of tOE17.

Productive Binding Can Be Competed by Delta pH Pathway Substrates—If precursor binds to a specific site related to the Delta pH pathway, its binding should be competed by substrates of the Delta pH pathway. This was tested by assaying the binding of radiolabeled \( \text{in vitro} \) translated tOE17 in the presence of increasing concentrations of unlabeled \( \text{E. coli} \)-produced precursor proteins (Fig. 6). The presence of increasing concentrations of unlabeled tOE17 resulted in decreased binding of radiolabeled precursor (Fig. 6, A and B), although there remained residual binding that actually increased somewhat at high concentrations of competitor (Fig. 6B, ●). Importantly, the amount of productively bound precursor decreased dramatically with increasing competitor concentration (Fig. 6, A and B, ■). Productively bound precursor is that which is subsequently transported into the lumen during the chase reaction. Inhibition of productive binding followed a similar curve as inhibition of the import of freshly added precursor (Fig. 6B, ▲). This and other experiments indicated that tOE17 binds specifically to thylakoids but that a varying amount of nonproductive or non-specific binding also occurs. The amount of nonproductive binding was low in most experiments that contained only \( \text{in vitro} \) translated precursor but increased substantially when \( \text{E. coli} \)-produced precursors were present in the assay.

Similar results of competition for labeled tOE17 binding-chase and transport were obtained with unlabeled DT23 as...
with the fact that peted by overexpressed tOE17 (data not shown). This, along
25% the relative level of tOE17 and was not significantly com-
by recombinant methods, mOE17 bound to thylakoids at only
mOE17 because there is only one methionine in tOE17 that
varies in amount with the translation reaction, also binds to
(0 competitor). *bound precursor, ▲, mature OE17 transported during the chase reaction, A, mature OE17 produced in transport reactions with freshly added precursor.

competitor, although the extent of competition was less than when tOE17 was used as competitor (Fig. 6). pOE33, which is
a substrate for the thylakoid Sec pathway, did not compete for
tOE17 binding-chase or transport. The above results demon-
strate that elevated concentration of tOE17 saturates a mem-
brane component involved in precursor binding. Other experi-
ments (data not shown) verified that incubation with saturating levels of tOE17 or DT23 does not disable the Delta
pH transport system or impair the ability of the membranes to
maintain a pH gradient. Namely, transport of radiolabeled E.
coli-produced tOE17 was maximum at 1.25–2.0 μM, and mem-
branes that had previously been incubated with saturating concentrations of DT23 exhibited efficient transport of freshly added Delta pH pathway substrates.

In vitro translation of tOE17 frequently results in two OE17
species. One is tOE17, whereas the second migrates at the approximate location of mature OE17. This lower band, which
varies in amount with the translation reaction, also binds to
the thylakoids (Fig. 6A). The lower band is unlikely to be
mOE17 because there is only one methionine in tOE17 that
could serve as initiator. In a direct comparison of binding of
tOE17 and an authentic mOE17 translation product produced
by recombinant methods, mOE17 bound to thylakoids at only
25% the relative level of tOE17 and was not significantly com-
peted by overexpressed tOE17 (data not shown). This, along
with the fact that E. coli-expressed DT23 as well as tOE17, but
not pOE33, competed for binding of the lower band in Fig. 6,
suggests that it is a carboxyl-terminally truncated tOE17. It is
not clear how it is produced from the in vitro translation, but it
may result from early termination, which has previously been
observed with wheat germ translation.3

Anti-Hcf106 Prebinding to Thylakoids Reduces Their Ability
to Bind tOE17—Hcf106 was identified in maize as a necessary
component of the Delta pH pathway by both in vivo and in vitro
methods (11, 21). Tha4 is a homologous protein that is also
required for Delta pH pathway function (6, 12). We recently
showed that antibodies against Hcf106 and Tha4 proteins spe-
cifically inhibit transport of proteins on the Delta pH pathway
(6). We similarly showed that antibodies to cpSecY specifically
inhibit transport on the thylakoid Sec pathway (6). The effects
of antibodies to Hcf106, Tha4, and cpSecY on tOE17 binding
and the subsequent chase were examined by prebinding the
respective IgGs to thylakoids and using the resulting thyla-
koids in assays (Fig. 7).

Anti-Tha4, cpSecY, and preimmune IgGs had no effect on the
binding of tOE17, even though the Tha4 antibodies were active
in inhibiting transport of tOE17 and chase of bound tOE17
(Fig. 7A). Antibody to Hcf106 reduced the amount of tOE17
bound in this and in several other experiments. In the experi-
ment shown in Fig. 7A, binding to anti-Hcf106-treated thyla-
koids was only 44% of that which occurred to either untreated
thylakoids or pre-immune-treated thylakoids. In Fig. 7B, it
can be seen that inclusion of the antigen (hcf106sd) at 15 μM during
antibody preincubation prevented the inhibition of binding.
Anti-Hcf106 only partially inhibited binding, even though it
exerted a nearly complete inhibition of transport. Unfortu-
nately, it was not possible to determine whether the residual
binding was productive or nonspecific because of the presence
of transport-inhibiting antibodies. At least some of the binding
may have been nonspecific because only 75% and 60% of the
precursor bound to control thylakoids was subsequently chased
into the lumen in Fig. 7, A and B, respectively.

DISCUSSION

Because protein transport on the Delta pH pathway requires
no stromal components, it has been assumed that recognition

3 H. Mori and K. Cline, unpublished observations.
and initial interaction with the translocation machinery occur on the membrane. Previous attempts to trap a bound intermediate on the thylakoids were unsuccessful. Such attempts were largely confined to the most commonly studied substrate, iOE23, which displays very little if any stable binding to the thylakoids (Ref. 22; Fig. 3). By surveying a range of Delta pH pathway substrates, we identified several substrates, most of which are translocated strictly on the Delta pH pathway (Fig. 6). When Pftf integration was inhibited by dissipating the Delta pH across the thylakoids, a site associated with the Delta pH pathway, but it had no effect on the transport of the Sec pathway substrate, iOE33.4 Second, substrates that productively bind are more rapidly and efficiently transported than substrates that exhibit low levels of binding (Fig. 2).4 Finally, at least two authentic Delta pH pathway intermediates, iOE17 and iPftf, bind to thylakoids. The physiological intermediate iOE17 binds to thylakoids, is transported from the bound state (Fig. 3), and exhibits all of the characteristics of binding and chase that were shown for tOE17 (Figs. 1–6). Also, in a recent study, we showed that integration of the membrane protein Pftf is mediated by the Delta pH pathway.5 When Pftf integration was inhibited by dissipating the Delta pH, the physiological intermediate iPftf specifically bound to thylakoids.5

The precise meaning of anti-Hcf106 inhibition of tOE17 binding is not yet clear. The fact that other thylakoid-binding antibodies (6) had no effect says that it is not antibody coating of the membrane surface per se that was inhibitory, but rather antibody binding to Hcf106 that was important. Although the lack of effect of anti-Tha4 antibodies does not rule out Tha4 involvement in precursor binding, the reproducible inhibition by anti-Hcf106 argues that the Hcf106 protein plays some role in the binding process. One possibility is that Hcf106 directly binds the precursor. It has been suggested, based on its membrane topology, that Hcf106 functions as a receptor (11). Hcf106 and the homologous Tha4 are anchored in the thylakoids by single amino-proximal transmembrane helices, exposing larger carboxyl-terminal domains to the stroma. Indeed, the ability of anti-Hcf106 to inhibit binding and transport of precursors is analogous to the effects of antibodies to the chloroplast import receptor Toc159 (formally Toc86) on precursor binding and import into the chloroplast (23). However, it remains to be determined whether Hcf106 makes direct contact with Delta pH pathway precursors, in particular with their signal peptides.

Another possibility is that Hcf106 is a component of a translocation complex and that antibody binding to any member of the complex impairs the ability of the machinery to interact with precursors. In fact, recent studies in our laboratory suggest that Hcf106 plays some role in translocation other than or in addition to signal peptide recognition. Specifically, a form of

4 X. Ma and K. Cline, unpublished observations.
Phtf that lacks its signal peptide still depends on Hcf106 for its integration. If this latter model is correct, it might explain the incomplete inhibition of binding by anti-Hcf106 compared with the complete inhibition of transport (Fig. 7). Resolving the precise role of Hcf106 either as a receptor or in some other aspect of the targeting and translocation process can now be addressed as a result of studies reported here.

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