Stoichiometry for binding and transport by the twin arginine translocation system

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Twist arginine translocation (Tat) systems transport large folded proteins across sealed membranes. Tat systems accomplish this feat with three membrane components organized in two complexes. In thylakoid membranes, cpTatC and Hcf106 comprise a large receptor complex containing an estimated eight cpTatC-Hcf106 pairs. Protein transport occurs when Tha4 joins the receptor complex as an oligomer of uncertain size that is thought to form the protein-conducting structure. Here, binding analyses with intact membranes or purified complexes indicate that each receptor complex could bind eight precursor proteins. Kinetic analysis of translocation showed that each precursor-bound site was independently functional for transport, and, with sufficient Tha4, all sites were concurrently active for transport. Tha4 titration determined that ~26 Tha4 protomers were required for transport of each OE17 (oxygen-evolving complex subunit of 17 kD) precursor protein. Our results suggest that, when fully saturated with precursor proteins and Tha4, the Tat translocase is an ~2.2-megadalton complex that can individually transport eight precursor proteins or cooperatively transport multimeric precursors.

Introduction

The Tat (twin arginine translocation) system transports folded proteins across ion-tight membranes using the proton-motive force as the sole energy source (Berks et al., 2000; Müller and Klösgen, 2005; Lee et al., 2006; Cline and Theg, 2007). These unusual features differentiate Tat from most other protein translocation systems, which transport proteins in an unfolded conformation and are powered by nucleoside triphosphate hydrolysis. Tat systems transport substrates that vary in size from ~2 kD to >100 kD, meaning that the Tat protein–conducting structure has the ability to adjust its opening according to the passenger protein (Berks et al., 2000). Moreover, it must do this in a way that precludes uncontrolled ion (proton) leakage.

Tat can also transport precursor proteins that form oligomers. These remarkable feats are accomplished with only three membrane protein components: cpTatC, Hcf106, and Tha4 in thylakoids and the orthologous TatC, TatB, and TatA in prokaryotes. The components are found in two separate complexes in the membrane. cpTatC and Hcf106 form a large receptor complex (Cline and Mori, 2001). Site-directed cross-linking studies identified cpTatC (TatC) as the primary receptor for the twin arginine signal peptide (Alami et al., 2003; Gérard and Cline, 2006). The receptor complex has been characterized in detergent extracts as a heterooligomer of 500–700 kD that contains only cpTatC-Hcf106 (TatC-TatB) in a 1:1 molar ratio (Bolhuis et al., 2001; Cline and Mori, 2001; McDevitt et al., 2006). This and other considerations (see Discussion) suggest approximately eight copies of each component per complex. The estimated number of cpTatC-Hcf106 pairs per complex suggests that each receptor complex could potentially bind approximately eight precursor proteins. However, this question is currently unresolved. Single-particle electron microscopic analysis of purified Escherichia coli receptor complexes showed a maximum of two precursor proteins per complex (Tarry et al., 2009). In contrast, Ma and Cline (2010) showed evidence for at least four precursors bound to an individual receptor complex.
Table 1. Reevaluation of Tat components in pea chloroplasts and thylakoids

| Protein | Intact chloroplasts (molecule/cp) | Thylakoids (molecule/thylakoid) | Stoichiometry |
|---------|----------------------------------|---------------------------------|--------------|
|         | Mean  | SD  | Mean  | SD  | Chloroplasts | Thylakoids |
| cpTatC  | 8,800 | 400 | 9,700 | 200 | 1            | 1          |
| Hcf106  | 39,000| 2,000|37,700 | 1,800| ~4           | ~4         |
| Tha4    | 186,500| 12,900|127,900| 31,700| ~21          | ~13        |

The molecules of cpTat components per chloroplast and thylakoid were determined by quantitative immunoblotting using full-length IVT protein standards (see Materials and methods, Fig. S1, and Tables S1 and S2) and are the means and SDs of three biological replicates. Chloroplasts and washed thylakoids were isolated from pea seedlings (see Materials and methods).

Results

Methodology

The tOE17 V-20F precursor protein is an OE17 precursor modified with a phenylalanine at the twin arginine (RR) +2 position, resulting in higher affinity binding (Ma and Cline, 2000; Gérard and Cline, 2007). The RR +2F motif is found in at least one cpTat substrate (Summer et al., 2000) and most bacterial Tat substrates, where it plays an important role in transport efficiency (Stanley et al., 2000). The binding affinity of tOE17 V-20F, although greater than most cpTat substrates, allowed analysis of binding stoichiometry with intact membranes and with in vitro translated (IVT) precursor protein, which exhibits a very low level of nonspecific binding (Ma and Cline, 2000). In the figures and discussion that follow, this precursor will be referred to as tOE17 or RR-tOE17. A nonfunctional twin lysine variant of this precursor is referred to as KK-tOE17.

Accurate quantification of tOE17 and cpTat components was crucial to this study. IVT proteins were quantified by scintillation counting, with appropriate corrections for the specific radioactivity of the tracer amino acid in the translation reaction (Fig. S1) and recovery of proteins from gel slices (see Materials and methods; Table S1). A novel and accurate immunoblotting method was developed to quantify cpTat components. It uses full-length IVT proteins as immunoblot standards (Fig. S1 and Table S2). Previous studies using aqueous soluble peptide antigens as immunoblotting standards reported a relatively low ratio of Tha4 to cpTatC (Mori et al., 2001; Jakob et al., 2009). We found that peptide standards dramatically overestimate cpTatC (Fig. S2) and thus underestimate the Tha4 per cpTatC stoichiometry. Our improved method produced a revised stoichiometry of 21:1 for Tha4/cpTatC in pea chloroplasts and somewhat less in washed thylakoids (Table 1), which supports the proposed role of Tha4 (TatA) as a channel component and further supports conclusions that Tha4 is easily removed from thylakoids during isolation and buffer washes (Frielingsdorf et al., 2008).

Precursor proteins bind to the Tat receptor with rapid association and a very low rate of dissociation

Relative rates of association and dissociation of tOE17 were determined before analysis of binding stoichiometry. Binding of tOE17 to the receptor complex occurred faster than we could practically determine with our assay, i.e., <5 min. Because binding reactions were performed for 60 min, equilibrium was assured even with low concentrations of tOE17. Dissociation of
As shown in Fig. 2A, pretreatment with antibodies to cpTatC, the primary receptor component (Alami et al., 2003; Gérard and Cline, 2006), reduced precursor protein binding to \( \leq 10\% \) of that with mock-treated membranes (Fig. 2A, lane 6). This reduction did not occur if the cpTatC antigen was included in the antibody-binding reaction (Fig. 2A, lane 5). Similarly, protease pretreatment of the membranes reduced binding to \( \leq 8\% \) of mock-treated membranes (Fig. 2A, lanes 7 and 8). Consistent with these values, binding of the nonfunctional KK-tOE17 was \( \leq 7\% \) of RR-tOE17 binding and was unaffected by antibody or protease pretreatment (Fig. 2B, lanes 2–8). Thus, with the conditions used to measure binding in this study, a low percentage of RR-tOE17 binds nonspecifically to the lipid bilayer. Furthermore, binding of KK-tOE17 is a representative measure for nonspecific binding.

Stoichiometry of tOE17 per cpTatC determined from saturation-binding isotherms

Saturation binding experiments were performed as a first approach to determine precursor protein binding stoichiometry to cpTatC. Thylakoids recovered from each binding reaction were analyzed by SDS-PAGE/fluorography to quantify tOE17 (Fig. 3A) and SDS-PAGE/immunoblotting to quantify cpTatC (e.g., see Fig. S1 and Materials and methods). The expected binding saturation curve was obtained but with gradually increased binding at higher tOE17 concentrations (Fig. 3C). Nonspecific binding, estimated with KK-tOE17 (Fig. 3, B and C), was linear in the concentration range used in the assay. Subtracting nonspecific binding at each concentration from the RR-tOE17 binding data produced a specific binding curve.

As shown in Fig. 2A, pretreatment with antibodies to cpTatC, the primary receptor component (Alami et al., 2003; Gérard and Cline, 2006), reduced precursor protein binding to \( \sim 10\% \) of that with mock-treated membranes (Fig. 2A, lane 6). This reduction did not occur if the cpTatC antigen was included in the antibody-binding reaction (Fig. 2A, lane 5). Similarly, protease pretreatment of the membranes reduced binding to \( \sim 8\% \) of mock-treated membranes (Fig. 2A, lanes 7 and 8). Consistent with these values, binding of the nonfunctional KK-tOE17 was \( \sim 7\% \) of RR-tOE17 binding and was unaffected by antibody or protease pretreatment (Fig. 2B, lanes 2–8). Thus, with the conditions used to measure binding in this study, a low percentage of RR-tOE17 binds nonspecifically to the lipid bilayer. Furthermore, binding of KK-tOE17 is a representative measure for nonspecific binding.

**Nonspecific binding**

Binding specificity was examined in several ways: binding of the nonfunctional twin lysine precursor protein KK-tOE17, binding to thylakoids pretreated with antibodies to cpTat receptor components, and binding to membranes pretreated with protease. Binding reactions were conducted with concentrations of precursor protein that would saturate the binding sites.
Because in practice cooperativity is most evident in the middle region of the binding isotherm, a binding experiment with additional data points in this region of the curve was conducted and the data subjected to Hill analysis, which gave a Hill coefficient of 1.18 (Fig. S3). This indicates that there is no cooperativity of binding, i.e., no interaction between sites in the receptor complex.

The increase in binding sites that result from importing precpTatC into chloroplasts is approximately one precursor per imported cpTatC. Additional approaches measured the tOE17/cpTatC binding stoichiometry at saturation. The first is based on previous observations that cpTatC imported into purified chloroplasts exhibits all of the characteristics of endogenous cpTatC (Fincher et al., 2003). IVT radiolabeled precpTatC (precursor to cpTatC) was imported into chloroplasts, and the thylakoids isolated from those chloroplasts were used for saturation-binding reactions (Fig. 4 A). The increase in binding was obtained as the difference in the amount of bound tOE17 between thylakoids with imported (imp.) cpTatC and thylakoids from the mock import reaction. Error bars represent the mean ± SD (n = 3). Chl, chlorophyll.

The Hill coefficient for the four binding curves was 1.09 (SD 0.18), as determined by curve fitting to a binding isotherm equation containing a Hill parameter (see Materials and methods). Because in practice cooperativity is most evident in the middle region of the binding isotherm, a binding experiment with additional data points in this region of the curve was conducted and the data subjected to Hill analysis, which gave a Hill coefficient of 1.18 (Fig. S3). This indicates that there is no cooperativity of binding, i.e., no interaction between sites in the receptor complex.

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Stoichiometry of tOE17 per cpTatC with purified precursor protein-bound cpTat receptor complexes

A second alternative approach involved saturating thylakoid membranes with tOE17, solubilizing the membranes with digitonin, and then purifying the receptor complexes by affinity chromatography. Two different but complementary affinity purification procedures were used, one based on affinity to a receptor component and the other based on affinity to the precursor protein. In Fig. 5 (A and C), the solubilized complexes were purified by immunoaffinity to anti-Hcf106 IgG immobilized beads (see Materials and methods). Only small amounts of tOE17 and cpTatC were present in the flow-through and wash fractions, and the total recovery of tOE17 was 85% (Fig. 5, A and C). The stoichiometry of tOE17 to cpTatC in the eluate was 0.79 (SD 0.18; n = 3). In the second affinity method, membranes were saturated with a C-terminally His-tagged tOE17 (tOE17-GS3-His), and solubilized complexes were purified by metal ion affinity chromatography (see Materials and methods; Fig. 5, B and D). Only small amounts of cpTatC and trace amounts tOE17-GS3-His were detected in the flow-through and wash fractions. The overall recovery of tOE17-GS3-His was 88%, and the ratio of tOE17-GS3-His to cpTatC in the eluate was 1.05 (SD 0.22; n = 3). The mean for the two affinity methods is 0.92 tOE17/cpTatC.

To summarize, the ratio of tOE17 per cpTatC obtained by the three different approaches—binding saturation curves, increase in binding after cpTatC import, and purification of fully occupied complexes by affinity chromatography—was 1.27, 0.92, and 0.92, respectively. The mean of the three methods, 1.04, indicates a stoichiometry of one tOE17 per cpTatC at saturation and implies that the receptor complex can bind up to eight precursor proteins (see Discussion).

All binding sites are functional for transport

Transport of receptor bound precursor protein (chase) can be modeled as a two-step reaction. In the first step, Tha4 assembles with the precursor-bound receptor. In the second step, the precursor is transported and the signal peptide cleaved by the luminal signal peptidase to produce the mature protein. Mori and Cline (2002) used chemical cross-linking during a chase reaction to show that Tha4 assembly correlates with an initial lag phase and is temporally distinct from the transport phase. Furthermore, Tha4, which dissociated after completion of transport, reassembled when more precursor protein was added to the reaction (Mori and Cline, 2002). A simple equation for this process is

\[ P + R + nT4 \rightarrow k_s \rightarrow P + R + nT4 + M \]

where P denotes precursor protein, R denotes receptor, T4 denotes Tha4, M denotes the transported mature protein, \( k_s \) denotes the assembly rate constant, and k denotes the translocon rate constant. To examine characteristics of transport in more detail, chase kinetics were analyzed with different sets of starting conditions that included tOE17 occupying \( \sim 20\% \) versus 100% of binding sites and a range of Tha4 concentrations from 6 Tha4 per tOE17 to 115 Tha4 per tOE17 (Fig. 6). The latter was obtained by supplementing reactions with IVT Tha4 (Dabney-Smith et al., 2003). Transport was scored by the appearance of the mature form of OE17 (mOE17; see Materials and methods). As shown in Fig. 6, in three out of four of the conditions (including one in which all sites were occupied with precursor protein), \( \sim 90\% \) of the initially bound tOE17 was transported in 25–30 min, which is consistent with \( \pm 90\% \) specific and \( \pm 10\% \) nonspecific binding (Fig. 2). This indicates that all binding sites are functional for transporting their bound precursor protein.

Transport kinetics of these three cases (Fig. 6, A–C) consisted of a lag phase followed by a rapid translocation phase. Our attempts to solve for both \( k_s \) and \( k \) for chase reactions were not feasible. This is probably because, in nearly all situations, the Tha4 concentration significantly changes during the assembly step in an undefined manner (see Materials and methods). However, for situations in which [Tha4] is sufficient to functionally assemble all of the bound precursor sites (i.e., \([P] = [P+R+T4])\), the rate of translocation equal to \( k_s \times [P] \) and \( k \) can be determined by a fit of either [tOE17] or [mOE17] versus time during the translocation step (see Materials and methods).
The translocation step was separately analyzed by subtracting the lag time (Fig. 6) from chase datasets (see Materials and methods). For three of the chase reactions shown in Fig. 6, in which the Tha4/tOE17 was 24, 39, and 115, the data fit an exponential first-order kinetic model (Fig. 7). When the data were plotted as the percentage of mOE17 produced by the end of each reaction (i.e., to normalize differences in the initial [tOE17]), the three curves could be virtually superimposed (Fig. 7) and yielded very similar $k_t$ values of $\sim 0.3\ min^{-1}$. This suggests that $\sim 25$ Tha4/tOE17 are sufficient to functionally assemble a transport site. In support of this, the $k_t$ values for the translocation phase in experiments in which Tha4 per tOE17 was $\geq 25$ were $0.27\ min^{-1}$ (SD $0.04; n = 5$) for tOE17-saturated receptors and $0.31\ min^{-1}$ (SD $0.05; n = 5$) for low-occupancy receptor complexes. T test analysis indicates that these values are not statistically different. The nature of first-order kinetics, in this case rate $= k_t \times [tOE17]$, is that all precursor protein-bound sites have an equivalent probability of transporting their bound precursor (Fig. 7). This does not mean that all sites transport simultaneously but that all sites are independently active for transport. Although the $k$ value of $\sim 0.3\ min^{-1}$ (approximate translocation rate) is rather slow for a protein translocation reaction, these assays were conducted at 15°C (to facilitate sampling); increasing the temperature of the chase reaction to 25°C increased the $k_t$ value at least twofold, resulting in $\tau = \sim 1.5$ min (unpublished data).

In the fourth chase reaction, the receptor complexes were saturated with precursor protein, but the Tha4/tOE17 was only six (Fig. 6 D).
falling off (Fig. 7). This would be consistent with a situation in which the available Tha4 functionally assembled only a fraction of the tOE17-bound sites initially and, after transport of those precursor proteins, recycled to new tOE17-bound sites (Fig. 7).

The minimal Tha4 oligomer required to transport an OE17 precursor has ~26 protomers

If this interpretation is correct, it should be possible to more precisely titrate the Tha4/tOE17 ratio to find the minimal Tha4/tOE17 to functionally assemble all of the tOE17-bound sites. This was accomplished with a series of chase reactions with tOE17-saturated membranes in which the Tha4/tOE17 was varied from 6 to 80 with IVT Tha4 (Fig. 8). The highest instantaneous rate of transport for each chase reaction was determined from the first derivative of a sigmoidal fit of the data. The mean sufficiency point obtained from two independent repeats with 21–80 Tha4/tOE17 are shown. The intercept of the two lines [arrow] represents the Tha4 sufficiency point (21 Tha4 per precursor) for transport of tOE17. The mean sufficiency point for the three independent measurements was 26 (SD 6.3). [D] Lag times were estimated, as in Fig. 6, and plotted versus Tha4/tOE17. Data points in C and D are colored and correspond to the chase reactions shown in A and B. The plots of data in the figure are from a single representative experiment out of three independent repeats.

The Tha4 oligomer required to transport an OE17 precursor contains ~26 Tha4 protomers

The minimal Tha4 oligomer required to transport an OE17 precursor has ~26 protomers. Chase reactions with tOE17-saturated membranes were conducted with different amounts of IVT Tha4 (see Materials and methods). [A] Transport of tOE17 was quantified as the amount of mOE17 produced and plotted as the percentage of tOE17 at time 0, and the data were fit to a sigmoidal equation (see Materials and methods). [B] Instantaneous transport rates for each chase reaction were estimated with the first derivative of the fitted curves. [C] The amount of Tha4 (quantitative immunoblotting) per precursor protein (radiolabel) was estimated for each chase reaction (see Materials and methods) and plotted versus the highest instantaneous transport rates (Max rate). Regression lines for the chase reactions with 2, 25, 50, and 100 Tha4/tOE17 were shown. The intercept of the two lines [arrow] represents the Tha4 sufficiency point (21 Tha4 per precursor) for transport of tOE17. The mean sufficiency point for the three independent measurements was 26 (SD 6.3). [D] Lag times were estimated, as in Fig. 6, and plotted versus Tha4/tOE17. Data points in C and D are colored and correspond to the chase reactions shown in A and B. The plots of data in the figure are from a single representative experiment out of three independent repeats.

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The minimal Tha4 oligomer required to transport an OE17 precursor has ~26 protomers. Chase reactions with tOE17-saturated membranes were conducted with different amounts of IVT Tha4 (see Materials and methods). [A] Transport of tOE17 was quantified as the amount of mOE17 produced and plotted as the percentage of tOE17 at time 0, and the data were fit to a sigmoidal equation (see Materials and methods). [B] Instantaneous transport rates for each chase reaction were estimated with the first derivative of the fitted curves. [C] The amount of Tha4 (quantitative immunoblotting) per precursor protein (radiolabel) was estimated for each chase reaction (see Materials and methods) and plotted versus the highest instantaneous transport rates (Max rate). Regression lines for the chase reactions with 2, 25, 50, and 100 Tha4/tOE17 were shown. The intercept of the two lines [arrow] represents the Tha4 sufficiency point (21 Tha4 per precursor) for transport of tOE17. The mean sufficiency point for the three independent measurements was 26 (SD 6.3). [D] Lag times were estimated, as in Fig. 6, and plotted versus Tha4/tOE17. Data points in C and D are colored and correspond to the chase reactions shown in A and B. The plots of data in the figure are from a single representative experiment out of three independent repeats.

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which is within the variance of the 26.4 (SD 6.3) obtained in the chase reactions supplemented with IVT Tha4 (Fig. 8).

The lag phase is dependent in part on the relative Tha4 concentration

As described in the previous section, the Tha4 assembly phase of the reaction is thought to involve Tha4 diffusion to and assembly with precursor-bound sites. A consistent relationship was found for lag time versus Tha4/tOE17 (Figs. 8 D and 9 D). In both experimental approaches, lag time decreased with increasing highest instantaneous rates, and a straight line was drawn for the two chase reactions that had achieved the maximum rate. (B and C) The intercept between the two lines indicates the Tha4 sufficiency point (arrows). (D) The lag time for each chase reaction was plotted versus the corrected Tha4/tOE17 ratio. The data points in B, C, and D are colored and correspond to the chase reactions shown in A. The data are from a single representative experiment out of two independent repeats. The corrected Tha4/tOE17 sufficiency point for the two experiments was 31.

Discussion

This study addressed the stoichiometry of precursor protein binding to the cpTat receptor complex and the stoichiometry of Tha4 required for transport. Three different methods were used to determine the binding stoichiometry of precursor protein per cpTatC. These methods, using both intact membranes and purified membrane complexes, gave remarkably similar ratios of approximately one bound tOE17 per cpTatC, providing strong support for a 1:1 stoichiometry. This suggests that each receptor complex can concurrently bind approximately eight precursor proteins, based on the estimated number of cpTatC per receptor complex. The isolated thylakoid receptor complex contains only cpTatC and Hcf106 (unpublished data), similar to the E. coli TatBC complex (McDevitt et al., 2006; Orriss et al., 2007), and migrates on blue native PAGE at ~700 kD. After correcting for the contribution of bound Coomassie blue to the apparent molecular mass (Heuberger et al., 2002), the estimated number of cpTatC-Hcf106 heterodimers is ~7.5 per complex, which compares well with structural results for the E. coli TatBC receptor complex (Tarry et al., 2009). Analysis of the transport step suggests that all precursor protein-bound receptor sites can be individually activated for transport and, furthermore, suggested a minimal Tha4 oligomer size of ~26 protomers for transporting an OE17 precursor protein. This implies that, for tOE17, a functional Tat translocase unit consists of one cpTatC (one Hcf106) and ~26 Tha4.

The binding stoichiometry obtained here is higher than that reported by Tarry et al. (2009), who found one to two precursor proteins per purified E. coli TatBC receptor complex. The discrepancy may be a result of dissociation of precursor proteins from the TatBC complex during purification procedures. The tOE17–cpTatC:Hcf106 complex was stable to the purification procedures used here (Fig. 5). However, we have previously observed precursor dissociation from the thylakoid receptor complex during electrophoresis on blue native polyacrylamide gels, the extent of which varied with the nature of the bound precursor (Cline and Mori, 2001; Gérard and Cline, 2007; Ma and Cline, 2010). Clearly, the stoichiometry obtained for purified receptor complexes will depend on the harshness of the purification method and the dissociation constants of each particular precursor protein. Binding of tOE17 exhibited a $K_d$ of ~1 nM and very slow dissociation at 0–15°C, whereas the precursors used by Tarry et al. (2009) have apparent $K_d$ values of 50–100 nM, although recent estimates from a fluorescence resonance energy transfer–based assay suggest a $K_d$ for preSufI of 7–23 nM (Whitaker et al., 2012).
the low number of preSUfI and CueO precursors that Tarry et al. (2009) recovered with purified complexes.

The high affinity of tOE17 for the cpTat receptor minimized nonspecific binding to the membrane bilayer (Fig. 2), which increases linearly with precursor protein concentration (Fig. 3). Other binding studies with E. coli inverted vesicles (Bageshwar et al., 2009) and thylakoid membranes (Musser and Theg, 2000; Hou et al., 2006) have observed precursor protein binding to the lipid bilayer and have further suggested that binding to the bilayer is an intermediate in the binding pathway. Although we can’t exclude this possibility, our results here (Fig. 6) and in unpublished studies indicate that lipid-bound tOE17 present at the start of a chase reaction was not productive, i.e., it was not transported during the chase reaction. Nevertheless, lipid-bound precursor proteins did not impact the results and conclusions of the present study because it was a very low percentage of total binding and was effectively subtracted (Fig. 3) or eliminated (Figs. 4 and 5) from the analyses.

Multivalent receptors frequently exhibit cooperativity, wherein binding to one site alters the affinity of other sites in the complex. The issue of cooperativity for the Tat transport system has been a matter of debate. Tarry et al. (2009) argued for a negative binding cooperativity for the E. coli Tat system to explain, in part, the fact that TatBC complexes contained only one or two bound precursor proteins. Alder and Theg (2003) reported positive cooperativity for translocation of the OE17 precursor protein, although it was not determined whether this occurred at the binding step or the translocation step. On the other hand, a similar study of translocation of the OE23 precursor found no evidence for cooperativity (Cline et al., 1993). The present study specifically examined the binding step and found no evidence of cooperativity, as judged by a Hill coefficient of ~1 (Figs. 3 and S3). Although it is conceivable that the enhanced affinity of the tOE17 precursor obscured cooperative interactions, we think this is unlikely because of the quality of the data in the lower to middle regions of the binding curves (Figs. 3 and S3).

Analysis of the translocation of bound tOE17 (chase) gave considerable insight into the mode of cpTat transport and the involvement of Tha4. When Tha4 was present in excess, the translocation step was described by a first-order kinetic model in which the instantaneous rate of transport equals $k_{\text{max}} [\text{bound precursor}]$. More importantly, with Tha4 excess, the $k_0$ values determined for a wide range of receptor complex occupancies, from well below one precursor per complex (Fig. 9) to fully saturated complexes (Figs. 7 and 8), were statistically identical. This observation is most easily interpreted to mean that all precursor-bound sites are independently competent for transport and can be concurrently active for transport. That is not to say that all precursor proteins bound to a receptor complex are simultaneously transported, although simultaneous transport can apparently occur in certain cases (Ma and Cline, 2010). Rather, it says that all sites have the same probability of undergoing translocation. If, for example, only one binding site was active per complex at a time, there would be a sequential progression of transport around a complex, and transport should be linear with time for extended periods rather than exponential.

The relationship between the relative amount of Tha4 and translocation rate was especially informative. The observation that above a threshold Tha4/tOE17 value the transport rate was maximum and constant (Figs. 8 and 9) strongly suggests that the threshold value is the point at which Tha4 has functionally assembled all tOE17-bound sites for transport. Its value of ~26 Tha4/tOE17 can be interpreted to mean that a 26-Tha4 oligomer complex facilitates transport of one tOE17 precursor protein. Two previous studies reported a measure of Tha4 (TatA) oligomer size in a functional context. An in vitro disulfide cross-linking study produced oligomers up to 18 Tha4 via C-tail Cys residues when Tha4 was assembled with the translocase in transporting membranes (Dabney-Smith and Cline, 2009). Fluorescence imaging of E. coli TatA-YFP (Leake et al., 2008) observed spots of variable size in the Tat-transporting E. coli cells with a median spot size of 25 TatA-YFP. Limitations of the techniques in these studies (i.e., inefficiency of disulfide cross-linking and uncertainty regarding the number of precursor-bound sties per TatA-YFP spot) precluded an estimate of the transport-active oligomer size. However, it is interesting that the Tha4 (TatA) oligomer sizes obtained are similar to our functional estimate (e.g., Fig. 8) as well as the stoichiometry of Tha4 per cpTatC in chloroplasts (Table 1).

Our results showing that precursor protein binding is non-cooperative and that all sites can be independently transport active raise the question of what advantage Tat might gain by organizing receptor units into a multivalent complex. One possibility is that when Tha4 is limiting, Tha4 oligomers could move to adjacent sites on the receptor complex without dissociation and reassembly. This would explain the observation that at very low Tha4/precursor ratios, the transport rate was nearly constant (Figs. 7 and 9), consistent with an absence of extensive lag for Tha4 recycling. Such a mechanism would provide an efficiency advantage for the steady-state operation of the cpTat system during periods of rapid thylakoid biogenesis.

Oligomeric Tha4 (TatA) is considered to serve as the protein-conducting component of the Tat system, and emerging data indicate that TatA contacts the mature domain of the precursor (Panahandeh et al., 2008; Maurer et al., 2010; Fröbel et al., 2011). The functional Tha4 oligomer size is tantalizing to view in the context of models for Tat transport. One model is that Tha4 (TatA) assembles appropriately sized oligomeric channels through which the bound precursor protein crosses to the trans side of the membrane (Gohlke et al., 2005; Leake et al., 2008). Another is that Tha4 (TatA) oligomers destabilize the bilayer structure and facilitate bound precursor protein movement across the membrane (Brüser and Sanders, 2003), possibly similar to the way that amphipathic antimicrobial peptides function (Tossi et al., 2000). If proteins are transported through structural channels, the size of the oligomer should be tightly linked to the molecular dimensions of the protein substrate but relatively unaffected by other assay parameters. Gohlke et al. (2005) estimated that 19 TatA protomers would provide a 30–35-Å channel, sufficient for the OE17 precursor. If the substrate protein is transported through the Tha4 destabilized membrane bilayer, the oligomer size may not be as dependent on substrate dimensions but instead sensitive to...
temperature and other factors that influence the fluidity and stability of the membrane, such as the presence of nonbilayer-forming lipids or the degree of saturation of the polar lipids. Of interest, a study of thylakoid transport characteristics in fatty acid desaturase mutants of Arabidopsis found that in membranes with increased fatty acid saturation (decreased fluidity), the activity of the cpTat system was decreased, whereas the activity of the cpSecA-SecYE system was increased (Ma and Browse, 2006).

Future studies to determine the relationship between the functional size of the Tha4 oligomer, precursor protein dimensions, composition of the membranes, and other parameters of the assay should provide considerable insight into the mechanism of protein translocation by the Tat system. At present, the translocation step is the most poorly understood aspect of Tat transport.

Materials and methods

Source plants, chloroplasts, and thylakoid isolation

Intact chloroplasts were isolated from 9- to 10-d-old pea seedlings (Progress #9 Improved; Buckeye Seed & Supply) and were resuspended in import buffer [IB; 330 mM sorbitol and 50 mM Hepes-KOH, pH 8.0; Cline et al., 1993]. Isolated thylakoids were obtained from intact chloroplast by osmotic lysis followed by centrifugation and were washed and resuspended in IB containing 5 mM MgCl₂ (IBM). Chlaphyll concentrations were determined according to Amon (1949). There are 10⁷ peptide chloroplasts or thylakoids per microgram of chlorophyll (Chen et al., 1990; Cline et al., 1993), and the thylakoid network remains intact after chloroplast lysis.

Plasmid construction and mutagenesis

Transcription clones for tOE17 V-20F (Gérard and Cline, 2007), pre-cpTatC and mcpTatC (mature cpTatC; Mori et al., 2001), and mTha4 (Fincher et al., 2003) are as previously described. The DNA clones for the His-tagged TOE17 precursor and His-tagged cpTat components (cpTatC, Hcf106, and Tha4) were constructed by PCR mutagenesis using the QuikChange kit (Agilent Technologies) by adding an unstructured (cpTatC, Hcf106, and Tha4) were constructed by PCR mutagenesis using the QuikChange kit (Agilent Technologies) by adding an unstructured

Preparation of radiolabeled precursor proteins

Capped RNAs were transcribed with SP6 polymerase (Promega) and translated in the presence of [³H]-Leu with a homemade wheat germ (WG) translation system (Cline, 1986). Translation reactions were incubated for 1 h at 25°C and stopped with 1 vol of 2x IBM containing 400 μM cycloheximide.

Quantification of IVT radiolabeled proteins

The specific radioactivity for [³H]-Leu in translation reactions was determined for each WG translation preparation by isotopic dilution experiments. A series of translation reactions were performed with a fixed amount of [³H]-Leu and increasing amounts of unlabeled Leu in a range from 0-80 μM final concentration (Fig. S1). Radiolabeled translation products were extracted from SDS-PAGE/fluorography gel slices (Cline, 1986), and dpm were measured by scintillation counting in a scintillation counter (LS6500; Beckman Coulter) calibrated for tritium and chemical and color quench. The reciprocal of the radioactivity was plotted against the total known Leu concentration in the reaction (added unlabeled Leu + [³H]-Leu), yielding the concentration of unlabeled Leu in the WG extract as the negative y-axis intercept (Fig. S1; Patrick et al., 1989). The nanomoles of translated precursor protein were determined using the specific radioactivity of Leu in the translation reaction, the dpm of the gel-extracted protein, a correction for the number of Leu residues per protein, and a correction for the extraction efficiencies determined for each protein (Table S1).

Quantitative immunoblotting

Samples received 1 vol of sample buffer (4% SDS, 66% glycerol, 5% β-mercaptoethanol, 2 mM EDTA, and 125 mM Tris, pH 6.8) and were incubated at 37°C for 20 min. Samples were analyzed on gels containing the minimum percentage of acrylamide to maximize electrophoresis transfer (9% acrylamide gels for cpTatC and Hcf106 and 12.5% for Tha4). Samples were loaded in gel lanes with a syringe. Gels were electroblotted to 0.2-µm pore-sized polyvinylidene fluoride membranes (cpTatC) or 0.25-µm pore-sized nitrocellulose membranes (Hcf106 and Tha4) for 60 min in a wet tank system with 200 mM glycine, 25 mM Tris, and 20% methanol. Blots were incubated with primary antibodies for cpTatC, Hcf106, and Tha4 (Mori et al., 1999; Mori et al., 2001) and developed with the ECL method (GE Healthcare) according to the manufacturer’s instructions. Immunoblots were scanned at 600 dpi per inch in transilient mode with a scanner (Perfection 3170; Epson) followed by image analysis by densitometry with Quantity One software (Bio-Rad Laboratories; Fig. S1 C). The amounts of the particular proteins were determined by comparison with full-length IVT protein standards (Fig. S1 C) and corrected for aggregation on gels (Table S1) and for inefficient electrophoresis of membrane-associated proteins (Table S2).

Chloroplast import and thylakoid protein integration assays

Radiolabeled prepCPtAC was incubated with isolated chloroplasts (0.33 mg chlorophyll/ml) and 5 mM Mg-ATP in IB with 120 μE/ml of light in a 25°C water bath for 30 min. After import, chloroplasts were treated with thermolysin and then purpurred by centrifugation through 10% Percoll cushion (Cline et al., 1993). Recovered chloroplasts were lysed hypotonically, and, thylakoids recovered by centrifugation were washed once and resuspended to 1 mg chlorophyll/ml. Integration of IVT Hcf106 and Tha4 into isolated thylakoids was performed by incubating translation mixtures with washed thylakoids (0.5 mg chlorophyll/ml) at 25°C in the dark for 20 min. After integration, membranes were washed twice and resuspended in IBM.

Precursor protein-binding assay

IVT precursor proteins were treated with apyrase (10 U per 100-μl translation) for 30 min on ice to remove nucleoside triphosphates. Binding reactions were performed by incubating precursor proteins with isolated thylakoids in the dark at 4°C with end-over-end mixing for 1 h. Binding reactions with different amounts of precursor protein were prepared by diluting IVT precursor with apyrerase-treated mock translation reaction mixture. Thus, all binding reactions contained an equal concentration of translation components. Typically, binding reactions contained 40 μg chlorophyll of thylakoids and 120 μl of precursor protein in low-renaturation microfuge tubes. After binding, the unbound supernatant and bound thylakoid fractions were recovered by centrifugation, and the thylakoids were washed twice, changing tubes each time. Resuspended thylakoids were mixed with 1 vol 2x SDS buffer without tracking dye and chlorophyll concentrations measured to adjust samples for equal loading.

Binding saturation data analysis

Specific binding curves were obtained by subtracting nonspecific binding, determined with KK-TOE17 in parallel assays, from total binding of RR-TOE17. Specific binding data were fit by nonlinear regression to a single-site binding equation, B = Bmax × [precursor]/(Kd + [precursor]), where B denotes the concentration of free precursor, [precursor] denotes the concentration of free precursor, and the fitting parameters Bmax and Kd denote the maximum binding and the dissociation constant, respectively, using LabPlot-GNU software. The Hill coefficient in binding saturation assays was determined in two different ways. Specific binding data were fit to a single-site binding equation including a Hill slope term [h], B = Bmax × [precursor]/(Kd + [precursor]²), where h is the Hill coefficient. Alternatively, the Hill slope was determined by plotting the log(B/(B - h)) versus log([precursor]), where h is the fraction of occupied sites ([specific binding/Bmax] + [precursor]) is the concentration of free unbound precursor protein in each binding reaction. The Hill coefficient is the slope of the regression line of the plotted data.

Precursor protein chase assay

For transport assays, bound precursor protein [chase], thylakoids recovered from binding assays equivalent to 25 μg of chlorophyll were further incubated on ice for 20 min in the dark with 75 μl containing varying amounts of apyrase-treated IVT unlabeled mTha4. mTha4 dilutions were made by apyrerase-treated mock translation extract. Reaction mixtures then received 75 μl of stromal extract and were transferred to a 15°C water bath for 5 min in the dark. Aliquots were removed as time 0 samples, and
chase reactions were initiated with Mg-ATP (12 mM final concentration) and ~100 μM white light at 15°C. At time points, 10 μl aliquots were mixed with SDS buffer, and heated at 95°C for 3 min. Samples were analyzed by SDS-PAGE and fluorography, and transport was quantified by scintillation counting of the OE17 and mOE17 bands. Two observations indicate that appearance of mOE17 accurately measures transport. Hashimoto et al. (1997) showed that the OE17 precursor is not processed until the entire protein is across the membrane. Second, time-course analysis in which reactions were terminated rapidly with MgCl2 (Reed et al., 1990) showed that all transported proteins had been processed to mature form (unpublished data). Chase reactions were conducted at 15°C rather than 25°C to slow the reaction, permitting sampling of more time points in the rapid phase of translocation.

Chase kinetics data analysis
The amount of mOE17 obtained for each time point was corrected for recovery and fit by nonlinear regression to a sigmoidal equation. Several sigmoidal models were attempted, including Boltzmann Sigmoidal, Sigmoidal dose response with variable slope (Prism 5; GraphPad Software), and a two-consecutive irreversible first-order reaction model (Conners, 1990). However, the two-step irreversible model only converged when [Tha4] was pseudoconstant, i.e., either a very high amount of Tha4 or a very low and recycling amount of Tha4. We found that all of the data points, especially in the lag phase, were best fit to a generic sigmoidal equation, y = a + (b - a) × x/[x + (c - x)], where y equals mOE17, x equals time, and a, b, and c are fitting parameters. The highest instantaneous transport rate in each chase reaction was obtained from the first derivative of the fitted sigmoidal curve by numerical differentiation using LabPlot-GNU. The lag time for each reaction was estimated by the x-axis intercept of the tangent line to sigmoidal curve at the point of highest instantaneous transport rate (Auer and Kashchiev, 2010). Lag times were subtracted from chase datasets, which were plotted and fitted to a first-order exponential equation, y = a × e-x/t + c, where y equals [mOE17], x denotes [OE17] present at time 0, k is the rate constant, and t is time. Similar methodology is described in Weiner et al. (1990) and Gettens and Gilbert (2007).

Solubilization and purification of precursor-bound receptor complexes
Thylakoids recovered from binding assays with saturating concentrations of precursor protein or His-tagged precursor protein were solubilized with digitonin, 0.5x IB, 20% glycerol, 0.5 M aminocaproic acid, and 2 mM PMSF, as previously described (Gérard and Cline, 2007). Insoluble material was removed by centrifugation at 150,000 g at 2°C for 30 min, and the supernatant was removed for subsequent affinity purification. For nonenaturing immunoprecipitation, the supernatant was incubated with His-106-JG cross-linked to protein A Sepharose beads and mixed end over end for 2 h at 4°C (Cline and Mori, 2001). Unbound proteins were recovered by centrifugation, and the beads were washed with 0.5% digitonin, 0.5x IB, 20% aminocaproic acid, and 20% glycerol for 10 min at 4°C. Bound proteins were recovered by incubating the beads with 2x SDS, 8 M urea, and nonreducing SDS buffer for 16 h at room temperature and removing the beads with a mini spin column (Promega).

For affinity purification to Ni–nitrilotriacetic acid magnetic agarose, the IgA-agarose beads (QIAGEN), the 150,000-g supernatant was combined with beads and 1 vol of 40 mM Heps, pH 7.8, 300 mM NaCl, and 40 mM imidazole and mixed end over end at 4°C for 8 h. Unbound proteins were recovered with a magnetic rack. The beads were washed with 20 mM Heps, pH 7.8, 150 mM NaCl, 20 mM imidazole, and 0.25% digitonin, and bound proteins were eluted with the same buffer containing 100 mM EDTA for 1 h with end-over-end mixing at 4°C. A second elution was performed with 2x SDS buffer containing 100 mM EDTA at room temperature for 1 h. Elution fractions were combined for analysis.

Image acquisition and processing
Images of radiolabeled proteins on polyacrylamide gels and of immunoblots were obtained by digital scanning of fluorogram films and immunoblot films with a scanner (Perfection 3170). Images were analyzed without additional processing and were prepared for figures without or with minimal adjustments of brightness and/or contrast.

Online supplemental material
Fig. S1 shows quantification of IVT radiolabeled proteins and endogenous ctpA proteins. Fig. S2 shows differential immunoblotting behavior between soluble domain antigens and full-length proteins. Fig. S3 shows a binding saturation assay and Hill slope analysis. Table S1 shows extraction efficiencies of radiolabeled proteins from dried gels. Table S2 shows dual-radiolabel immunoblot calibration. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201210996/DC1.

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