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Functional reconstitution of bacterial Tat translocation *in vitro*

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The Tat (twin-arginine translocation) pathway is a Sec-independent mechanism for translocating folded preproteins across or into the inner membrane of *Escherichia coli.* To study Tat translocation, we sought an *in vitro* translocation assay using purified inner membrane vesicles and *in vitro* synthesized substrate protein. While membrane vesicles derived from wild-type cells translocate the Sec-dependent substrate proOmpA, translocation of a Tat-dependent substrate, SufI, was not detected. We established that *in vivo* overexpression of SufI can saturate the Tat translocase, and that simultaneous overexpression of TatA, B and C relieves this SufI saturation. Using membrane vesicles derived from cells overexpressing TatABC, *in vitro* translocation of SufI was detected. Like translocation *in vivo*, translocation of SufI *in vitro* requires TatABC, an intact membrane potential and the twin-arginine targeting motif within the signal peptide of SufI. In contrast to Sec translocase, we find that Tat translocase does not require ATP. The development of an *in vitro* translocation assay is a prerequisite for further biochemical investigations of the mechanism of translocation, substrate recognition and translocase structure.

Keywords: membrane proteins/TatABCE/Tat translocase

Introduction

The transport of proteins across lipid bilayers is a fundamental process in all organisms. In *Escherichia coli*, most proteins are translocated across or integrated into the inner membrane by the Sec preprotein translocase. Since preproteins translocated by the Sec machinery must be in an unfolded state during translocation, they are unable to adopt tertiary structure prior to translocation. This raises an interesting question: how are proteins that bind cofactors in the cytosol, and therefore must have adopted some degree of tertiary structure, transported to the periplasm? Work in the last few years has demonstrated that bacteria have a dedicated translocation machinery, termed Tat (twin-arginine translocation), for this very purpose (Berks, 1996; Santini *et al*., 1998; Sargent *et al*., 1998; Weiner *et al*., 1998).

The Tat translocation pathway is a Sec-independent mechanism of translocating folded preproteins into or across the inner membrane of *E.coli.* Most substrates of the Tat translocase bind redox cofactors in the cytoplasm prior to translocation (Berks, 1996). The Tat translocase is also capable of translocating a folded heterologous (green fluorescent) protein to the periplasm in an active conformation (Thomas *et al*., 2001). Proteins are targeted to the translocase via an N-terminal signal peptide bearing a characteristic 'twin-arginine' motif [(S/T)RRxFLK] (Berks, 1996). Tat signal peptides are generally longer and less hydrophobic than Sec signal peptides (Cristobal *et al*., 1999). Each of these properties appears to be important for targeting proteins to the Tat translocase. Mutation of either arginine residue within the signal peptide results in a significant reduction in translocation efficiency (Cristobal *et al*., 1999; Stanley *et al*., 2000). In addition, increasing the hydrophobicity of the signal peptide has been reported to convert a Tat-dependent substrate into a Sec-dependent one (Cristobal *et al*., 1999).

A Tat-like translocase was first appreciated in plants as a protein import pathway of the chloroplast thylakoid membrane. The thylakoid pathway, termed ΔpH, translocates preproteins bearing twin-arginine signal peptides to the lumen of the thylakoid, driven by the pH difference across the thylakoid membrane (Mould and Robinson, 1991; Cline *et al*., 1992). Using a genetic screen in maize, a mutant was identified in which the ΔpH pathway was defective (Voelker and Barkan, 1995; Settles *et al*., 1997). The disrupted maize gene encodes a protein (HCF106) that is an essential component of the ΔpH translocase. Homologs of Hcf106 have been identified in a wide range of bacterial genomes (Dalbey and Robinson, 1999). Using a combination of genetic screens and homology searches, several groups established that homologs of Hcf106 are essential components of the Tat translocase in *E.coli* (Sargent *et al*., 1998; Weiner *et al*., 1998). *Escherichia coli* encodes three homologs of Hcf106, named TatA, B and E (Sargent *et al*., 1998; Weiner *et al*., 1998). All three are integral membrane proteins predicted to span the inner membrane once with their C-terminal domain facing the cytoplasm. TatA and E are the most closely related, and are functionally interchangeable (Sargent *et al*., 1998). TatB is more distantly related to TatA and E, and disruption of TatB alone is sufficient to abolish translocation of many Tat substrates (Sargent *et al*., 1999). TatA and TatB are part of an operon encoding TatA, B, C and D. The TatC protein has six transmembrane segments and has been proposed to function as the translocation channel and receptor for preproteins (Bogsch *et al*., 1998; Chanal *et al*., 1998; Berks *et al*., 2000a,b). A mutation in *tatC* blocks the export of at least five substrates of the translocase (Bogsch *et al*., 1998). Although encoded by the last gene of the *tatABCD* operon, TatD is thought to have no role in Tat translocation (Wexler *et al*., 2000). Little information is available regarding the spatial organization of the Tat proteins. A direct physical interaction between TatA and B has been demonstrated

by immunoprecipitation and gel filtration techniques (Bolhuis *et al*., 2000), and genetic interactions are observed between TatA and B and TatB and C (Sargent *et al*., 1999; Bolhuis *et al*., 2000).

We now report that overexpression of a model Tat substrate, SufI, saturates the Tat translocase and that simultaneous overexpression of TatA, B and C relieves this saturation. Using inverted membrane vesicles from cells overexpressing TatABC in an *in vitro* translocation assay, we show that translocation of SufI depends on the membrane potential, the twin-arginine signal motif and a functional translocase composed of TatABC.

Results

In the light of difficulties in detecting Tat translocation *in vitro* with membranes from wild-type *E.coli* (see below; Figure 6A, lane 3), we sought to optimize membranes for their capacity for Tat translocation. This was done through substrate saturation of the wild-type Tat translocase followed by assay for Tat proteins that had to be overexpressed to relieve this saturation. Many of the Tat substrates possess cofactor binding sites (Berks, 1996). Acquisition of the cofactor appears to be a prerequisite for translocation of the substrates investigated to date, with the exception of SufI (Berks, 1996; Santini *et al*., 1998; Stanley *et al*., 2000). Although SufI has homology to proteins of the multicopper oxidase superfamily, SufI is not thought to bind Cu^{2+} (Stanley *et al*., 2000). The absence of a cofactor simplifies studies of SufI translocation by eliminating the requirement for a cofactor insertion step. SufI is completely translocated across the inner membrane and released to the periplasm in a soluble form. Its small size (54 kDa) relative to other Tat substrates allows resolution of the precursor from the processed mature form on polyacrylamide gels. These properties make SufI an ideal model substrate for studies of Tat translocation (Stanley *et al*., 2000).

Translocation of SufI in vivo

To examine Tat-mediated translocation of SufI *in vivo*, we placed SufI expression under a T7 promoter. In addition, HSV and polyhistidine epitope tags were incorporated at the C-terminus, allowing detection of tagged SufI. Strains were grown in minimal media lacking methionine, induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 min, pulse-labeled with [^{35}S]methionine and chased with an excess of unlabeled methionine (Figure 1A, lanes 1–6). Maturation of SufI from the precursor form was linear over the first 5 min, by which time 80% of the substrate had been processed (Figure 1B). Similar kinetics were observed with untagged SufI (data not shown), demonstrating that the C-terminal epitope tags do not interfere with translocation. Processing of SufI was not detected in strains deleted for expression of TatC (Figure 1A, lane 7) or TatA or TatB (data not shown). Thus, SufI is processed in a Tat-dependent manner, and TatA, B and C are required for processing (Stanley *et al*., 2000).

Processing of SufI is sensitive to the membrane uncoupler carbonyl cyanide *m*-chlorophenol hydrazone (CCCP) (see Figure 5B) and to a specific β-lactam inhibitor of leader peptidase (Paetzel *et al*., 1998),

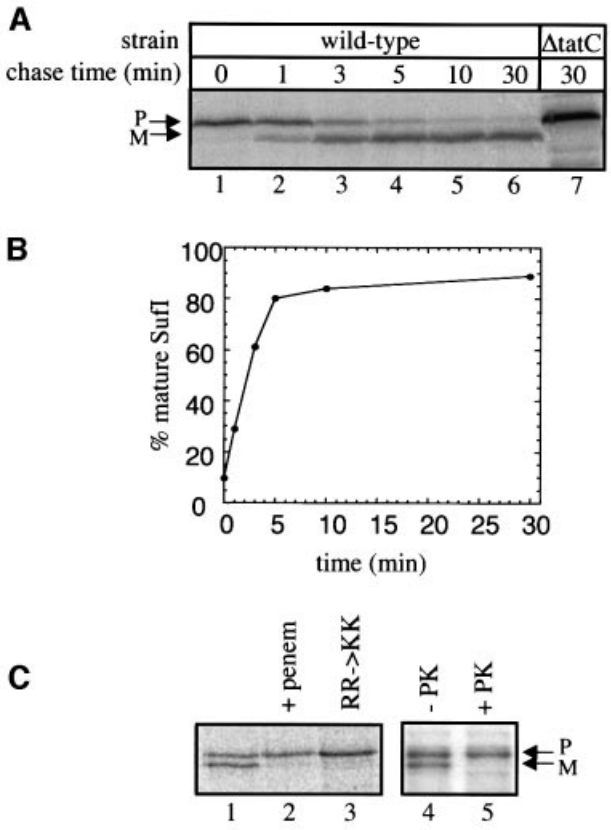

Fig. 1. (**A**) *In vivo* translocation of SufI. *Escherichia coli* MC4100(DE3) (lanes 1–6) or MC4100(DE3) ΔtatC (lane 7) carrying the pET-SufI expression plasmid was grown in M9 minimal media to A_{600} = 0.4. SufI expression was induced by the addition of IPTG to 1 mM for 3 min. Cells were pulse-labeled with [^{35}S]methionine for 1 min, then chased with methionine (500 μg/ml). At the times indicated, samples (400 μl) were mixed with 133 μl of cold 50% TCA, placed on ice and analyzed by adsorption to Ni-NTA–agarose, SDS–PAGE and fluorography as described in Materials and methods. The precursor (P) and mature (M) forms of SufI are indicated. (**B**) Quantitation of the data in (A). (**C**) The specificity of SufI translocation *in vivo*. *Escherichia coli* MC4100(DE3)ara^r carrying either pET-SufI (lanes 1 and 2 and 4 and 5) or pET-SufI(RR to KK) (lane 3) was grown as described in (A) and SufI expression was induced with IPTG for 5 min. Samples (400 μl) in lanes 1 and 2 were treated with either DMSO (5% final) or the penem leader peptidase inhibitor (1 mM) for 3 min. Cells were then pulse-labeled for 1 min and chased for 10 min with methionine (500 μg/ml). Samples (lanes 1–3) were precipitated with TCA and analyzed by Ni-NTA adsorption, SDS–PAGE and fluorography. Following the 10 min chase, cells (400 μl) (lanes 4 and 5) were collected by centrifugation (2 min at 14 000 *g*), suspended in 500 μl of 50 mM Tris–HCl pH 7.5, 10% sucrose, 1 mM EDTA, and lysozyme was added to 500 μg/ml. Samples were incubated for 15 min on ice, followed by the addition of proteinase K (300 μg/ml, final; lane 5 only) and incubation continued for 60 min on ice. Spheroplasts were collected by centrifugation (4 min at 14 000 *g*), suspended in 500 μl of M9 minimal media and proteins precipitated by the addition of TCA to 12.5%. Samples were processed for Ni-NTA precipitation and analyzed by SDS–PAGE and fluorography.

demonstrating a direct role for leader peptidase in the processing of SufI (Figure 1C, lane 2). Processing is also prevented by mutation of both arginine residues in the signal peptide of SufI to lysine (Figure 1C, lane 3). Consistent with previous reports, these data demonstrate that translocation of SufI requires an intact membrane

potential and Tat signal peptide (Stanley *et al*., 2000), and that processing is mediated by leader peptidase.

To determine whether the processing of SufI is an accurate indicator of its export to the periplasm, cells expressing SufI were pulsed for 1 min with radiolabeled methionine, chased with unlabeled methionine for 10 min, converted to spheroplasts and treated with proteinase K. The processed form (i.e. translocated periplasmic form) is selectively degraded, while the unprocessed form (i.e. cytosolic) is inaccessible to proteolysis (Figure 1C, lanes 4 versus 5). Thus, the processing of pre-SufI occurs soon after its export and the degree of processing is an accurate indicator of export.

Saturation of the Tat translocase and its relief by the overexpression of TatABC

The efficiency of SufI processing decreased with longer times of SufI expression prior to the pulse–chase, suggesting that overexpression of SufI saturates the Tat translocase. To demonstrate saturation, SufI expression was induced for 5, 10, 20 or 40 min with IPTG (Figure 2A), followed by a pulse with radiolabeled methionine for 1 min and a chase with unlabeled methionine for 0, 5 or 10 min. Following a 5 min induction, 70% of the pulse-labeled SufI is found in the processed form after a 10 min chase. In contrast, IPTG induction times of 10, 20 and 40 min result in a progessive decrease in the fraction of SufI that is processed to the mature form (Figure 2A and B). Aliquots from samples for each time of SufI induction were also analyzed for SufI by immunoblots. Processing of SufI had an inverse correlation with the increased accumulation of SufI protein (Figure 2B), indicating that the translocase had become saturated.

Finding conditions of substrate saturation of Tat translocase allowed us to assay for factors whose overexpression restores the ability of the translocase to process SufI. Plasmids were constructed that expressed TatABCDE, or various combinations of these proteins, under the control of an arabinose-inducible promoter. To confirm that each of the plasmids expressed the correct proteins, strains were grown in minimal media to mid-log phase, induced with arabinose for 2 h, and analyzed by SDS–PAGE and immunoblotting with antibodies to TatA, B, C, E and SecY (Figure 3). While TatA, B, C and E expressed at wild-type levels were detectable upon longer exposure (data not shown), expression of TatA, B and C was at least 32 times greater when overexpressed from a plasmid construct (Figure 3, lane 1 versus 2–7). The overexpression of TatE, although substantial from each of the constructs, was somewhat diminished for the TatABCDE or TatABCE plasmid, for unknown reasons (Figure 3, lanes 2 and 3).

To determine whether overexpression of Tat proteins might relieve the saturation observed upon overexpression of SufI, cells carrying the SufI and Tat expression plasmids were grown in minimal media to mid-log phase. Expression of SufI and Tat proteins was induced by IPTG and arabinose for 45 min, and cells were pulsed for 1 min with radiolabeled methionine and chased with an excess of unlabeled methionine for 0, 5 and 10 min. Overexpression of SufI saturates the Tat translocase and thereby reduces the processing of SufI (Figure 4A, pBAD). In contrast, the co-expression of TatABCDE, TatABCE or

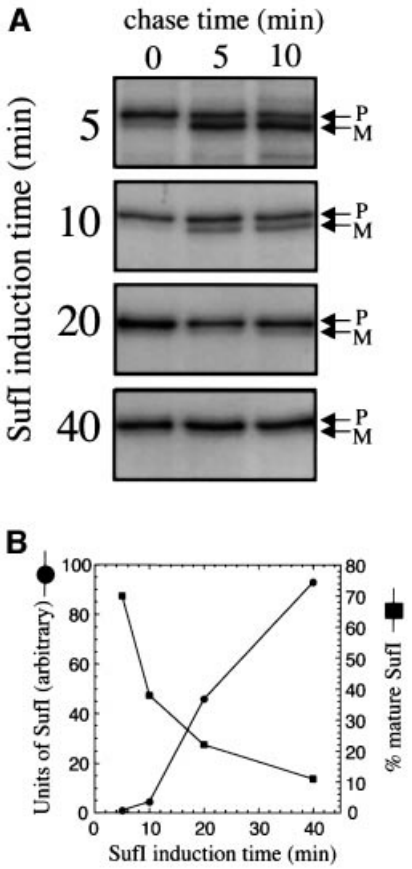

Fig. 2. (**A**) Saturation of the Tat translocase. MC4100(DE3)ara^r carrying pET-SufI was grown in M9 minimal media to A_{600} = 0.4. SufI expression was induced by the addition of IPTG to 1 mM. At 5, 10, 20 and 40 min post-induction, 1.4 ml samples were removed, pulse-labeled for 1 min with [^{35}S]methionine and chased with methionine (500 μg/ml). Aliquots (400 μl) were removed following 0, 5 and 10 min of chase, mixed with 133 μl of 50% TCA, and precipitated proteins were analyzed by Ni-NTA adsorption, SDS–PAGE and fluorography. (**B**) Quantitation of pre-SufI processing and SufI protein. The percentages of SufI in the mature form were determined for the 10 min chase. For each SufI induction time, an aliquot of the sample chased for 10 min was analyzed by SufI immunoblot analysis using antibody directed against the His_6 epitope tag. The protein content for each sample was normalized to the 5 min induction time (set to 1).

TatABC increases the processing of SufI (Figure 4A). These results are consistent with a previous study, which demonstrated that TatD plays no role in Tat translocation (Wexler *et al*., 2000). Deletion of either TatA, B or C from the Tat overexpression construct prevents the processing of SufI, demonstrating that each of these three components of the translocase is limiting *in vivo* under conditions of substrate excess. The inability to completely chase SufI from the precursor to the mature form following a 10 min chase suggests that either additional components may also be limiting under these conditions or that a fraction of the SufI may be in an export-incompetent conformation. As demonstrated in Figure 2, low levels of SufI expression correlate with an increased ability to process SufI precursor to the mature form. To eliminate the possibility that the increase in SufI processing observed in cells overexpressing TatABC was the result of decreased levels of SufI expression, samples from the 10 min chase were

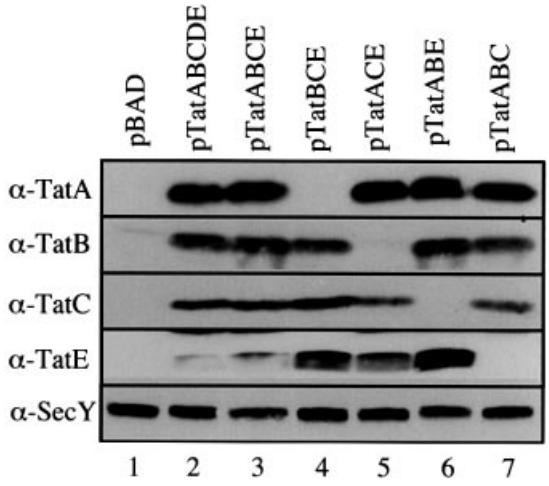

Fig. 3. Overexpression of Tat proteins. For analysis of Tat protein expression, MC4100(DE3)ara^r carrying either the parental vector pBAD (lane 1) or Tat expression plasmids pTatABCDE (lane 2), pTatABCE (lane 3), pTatBCE (lane 4), pTatACE (lane 5), pTatABE (lane 6) or pTatABC (lane 7) was grown in M9 minimal media to A_{600} = 0.3. Arabinose was added to 1% and incubation continued for 2 h. An equal number of cells was harvested from each culture, suspended in SDS–PAGE sample buffer, sonicated for 10 s, heated to 37°C for 10 min, and analyzed by SDS–PAGE [High-Tris (Brundage *et al*., 1990) for TatA and E, 15% for TatB, C and SecY] and immunoblot analysis with affinity-purified antibodies directed against TatA, B, C, E or SecY.

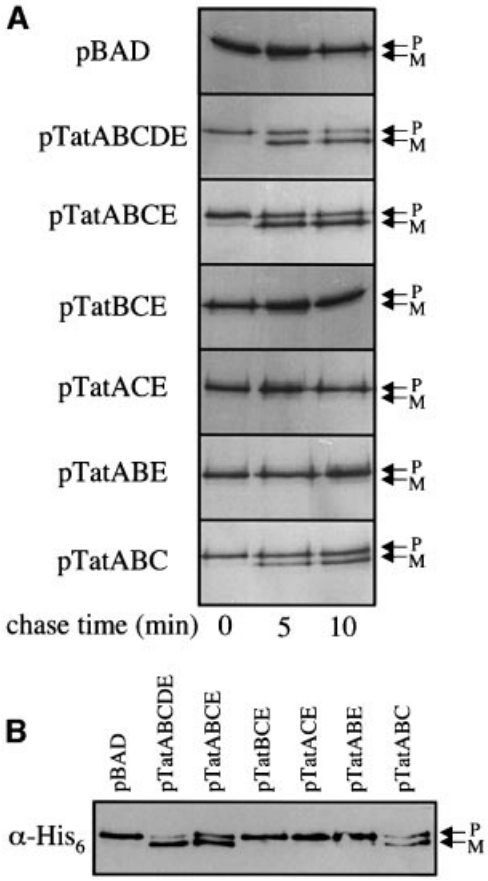

Fig. 4. (**A**) Overexpression of Tat proteins relieves the saturation of Tat translocase. MC4100(DE3)ara^r carrying pSU-SufI and either the parental vector pBAD or Tat expression plasmids was grown in M9 minimal media lacking methionine to A_{600} = 0.3. IPTG (1 mM final) and arabinose (1% final) were added and incubation was continued for 45 min. Samples (1.4 ml) were pulse-labeled with [^{35}S]methionine, chased with methionine (500 mg/ml) for 0, 5 and 10 min, and processed for Ni-NTA adsorption, SDS–PAGE and fluorography. (**B**) Immunoblot analysis of SufI protein. An aliquot from the 10 min chase from each of the samples in (A) was analyzed for SufI protein by immunoblotting using antibody directed against the His$_6$ epitope tag.

analyzed for SufI by immunoblotting. As seen in Figure 4B, the amount of SufI expressed is similar during induction of each Tat expression plasmid, suggesting that the relief of saturation is a direct consequence of TatABC overexpression.

SufI translocation occurs post-translationally

Substrates of the Tat translocase are thought to fold prior to translocation, suggesting that translocation proceeds post-translationally. This was important to confirm in the light of our goal of establishing an *in vitro* translocation assay. Strains carrying the SufI expression plasmid and either pBAD or pTatABC were grown in minimal media to mid-log phase, then induced for SufI expression for 45 min to saturate the translocase. Strains were pulse-labeled for 1 min with radiolabeled methionine and chased with an excess of unlabeled methionine for either 10 or 30 min. Immediately following the addition of unlabeled methionine, expression of TatABC was induced by the addition of arabinose. Under all conditions tested, strains carrying pBAD demonstrated <6% processing of the overexpressed pre-SufI to mature SufI (Figure 5A, lanes 1–4). However, strains carrying the TatABC plasmid, which had been induced with arabinose during the chase period, showed a 7-fold increase in the processing of precursor after 30 min (Figure 5A, lane 4 versus 8). Furthermore, there was a 2.5-fold increase in processing between 10 and 30 min of chase (Figure 5A, lane 6 versus 8), demonstrating that export of SufI can proceed post-translationally *in vivo*.

As an independent means of confirming this result, cells carrying the SufI expression plasmid were grown to mid-log phase and SufI expression was induced for 5 min. Cells were treated with either 5% dimethylsulfoxide (DMSO), the solvent for CCCP (Figure 5B, lanes 1 and 2), with CCCP (lanes 4 and 5) or with DMSO or CCCP pre-mixed with β-mercaptoethanol (Figure 5B, lanes 3 and 6, respectively) for 1 min. Cells were then pulse-labeled for 1 min with radiolabeled methionine and chased for 10 min with an excess of unlabeled methionine. CCCP caused a 3-fold reduction in SufI processing (Figure 5B, lane 1 versus 4). Pre-mixing CCCP and β-mercaptoethanol prior to the pulse-labeling neutralized the inhibitory effects of CCCP (Figure 5B, lane 6). The addition of β-mercapto-ethanol immediately after pulse-labeling reversed the inhibitory effects of CCCP and restored the processing of SufI (Figure 5B, lane 5), consistent with data in Figure 5A that SufI can be exported post-translationally.

In vitro translocation of SufI

The observations that overexpression of TatABC increases the efficiency of Tat translocation and that SufI may be exported post-translationally provided a starting point for the development of an *in vitro* assay for Tat translocation. Inverted membrane vesicles (IMVs) were prepared from

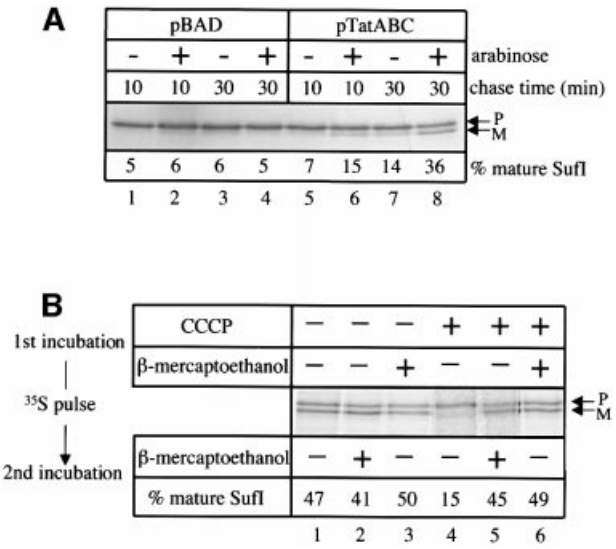

Fig. 5. (**A**) SufI post-translational translocation. MC4100(DE3)ara^r carrying pSU-SufI and either pBAD (lanes 1–4) or pTatABC (lanes 5–8) was grown in M9 minimal media to A_{600} = 0.4. Samples (0.9 ml) were pulse-labeled with [^{35}S]methionine for 1 min, followed by the addition of methionine (500 mg/ml) and 1% arabinose (lanes 2, 4, 6 and 8). Samples were incubated at 37°C for either 10 min (lanes 1 and 2 and 6 and 7) or 30 min (lanes 3 and 4 and 7 and 8) and 400 µl aliquots were processed for Ni-NTA adsorption, SDS–PAGE and fluorography. (**B**) Reversal of a CCCP block restores post-translational SufI translocation. MC4100(DE3)ara^r carrying pET-SufI was grown in M9 minimal media to A_{600} = 0.4. SufI expression was induced for 5 min with IPTG and samples were then treated with either DMSO (5%) (lanes 1 and 2), DMSO pre-mixed with β-mercaptoethanol (1 mM final) (lane 3), CCCP [100 µM (final) from a 2 mM stock in DMSO] (lanes 4 and 5) or CCCP pre-mixed with β-mercaptoethanol (lane 6) for 1 min, pulse-labeled with [^{35}S]methionine for 1 min and chased with methionine (500 µg/ml). Immediately following the addition of unlabeled methionine, β-mercaptoethanol (1 mM final) was added (lanes 2 and 5). Samples were incubated at 37°C for 10 min, transferred to ice and processed for Ni-NTA adsorption, SDS–PAGE and fluorography. Owing to the decreased incorporation of [^{35}S]methionine in the presence of CCCP, lanes 4 and 5 were exposed longer to achieve comparable signals.

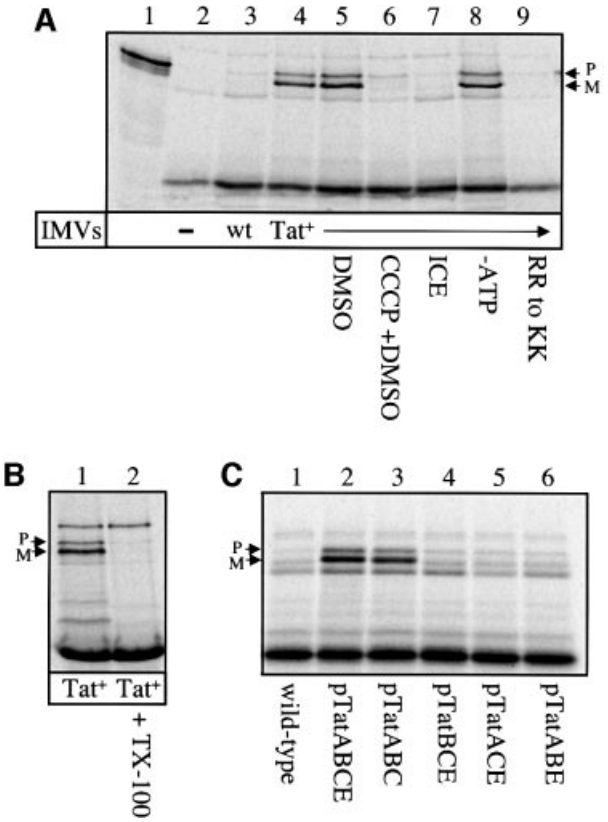

Fig. 6. (**A**) *In vitro* translocation of SufI. Complete translocation reactions (50 µl): lanes 3–7 and 9 contained 5 µl of 10 × TL buffer, 300 µg/ml IMVs [either wt (lane 3) or Tat-overexpressed (Tat$^+$, lanes 4–9)], 200 µg/ml lipid-free BSA, 2 mM ATP and 5 mM NADH. The reaction in lane 2 lacked IMVs and lane 8 lacked ATP and had 1 U of potato apyrase. Samples were incubated for 3 min at 37°C and DMSO (1% final) (lane 5) or CCCP (100 µM final from a 10 mM stock in DMSO) (lane 6) was added, followed by 20 µl of ^{35}S-labeled SufI (lanes 1–8) or SufI(RR to KK) (lane 9). Samples were incubated for an additional 60 min at 37°C (lanes 1–6 and 8 and 9) or on ice (lane 7). Reactions were transferred to ice and digested with proteinase K (1 mg/ml) for 15 min. Membranes were sedimented by centrifugation (10 min at 100 000 *g*), suspended in 100 µl of TL buffer, and protein was precipitated by the addition of TCA (12.5% final) and incubation on ice for 30 min. Precipitated protein was collected by centrifugation (at 14 000 *g* for 10 min at 4°C), mixed with 1 ml of acetone and sedimented, suspended in 40 µl SDS–PAGE sample buffer, heated at 95°C for 5 min, and analyzed by SDS–PAGE and fluorography. Lane 1 represents 0.5% of the SufI substrate in each translocation reaction. (**B**) Translocation assays were performed as described in (A), except that membranes were sedimented by centrifugation prior to digestion with proteinase K. During proteinase K digestion, Triton X-100 (1%) was added to the reaction in lane 2. (**C**) TatABC are required for *in vitro* translocation. Translocation assays were performed as in (A) containing wild-type, pTatABCE-, pTatABC-, pTatBCE-, pTatACE- or pTatABE-overexpression IMVs (lanes 1–6).

strains carrying either pBAD or the pTatABCE expression plasmid, which causes a 32-fold enrichment of TatABC in the whole cells (Figure 3) and isolated IMVs (data not shown) when compared with the vector control. Both wild-type and Tat-overexpression IMVs supported equivalent translocation of the Sec-dependent substrate proOmpA (data not shown).

SufI was synthesized in a coupled transcription–translation reaction with [^{35}S]methionine. Protein synthesis reactions were centrifuged to remove insoluble SufI and contaminating membranes. For *in vitro* translocation, reactions containing either wild-type or TatABCE-overexpression IMVs, ATP, NADH and bovine serum albumin (BSA) were pre-warmed to 37°C for 3 min, followed by the addition of radiolabeled SufI. Reactions were incubated for 60 min, treated with proteinase K on ice, and membranes were re-isolated, suspended, precipitated with trichloroacetic acid (TCA), and analyzed by SDS–PAGE and fluorography. In reactions either lacking membranes or containing wild-type membranes (Figure 6A, lanes 2 and 3, respectively), protease-inaccessible SufI was not detected. However, with TatABCE-overexpression membranes (lane 4), ~0.4% of the precursor and mature forms of SufI were reproducibly protected from added protease. Formation of the protected species of SufI is sensitive to the membrane uncoupler CCCP (lane 5 versus 6) or mutation of both arginines within the signal peptide to lysine (lane 9) and requires physiological temperature (lane 7). Depletion of ATP by the addition of apyrase has little effect on the formation of protease-inaccessible SufI, demonstrating that translocation does not require ATP (lane 8). Finally, to demonstrate that SufI has truly been translocated to the lumen of the vesicle, samples were

treated with Triton X-100. Following solubilization of the membranes with Triton X-100, the formerly inaccessible SufI is digested (Figure 6B, lane 1 versus 2), consistent with SufI being translocated. These data demonstrate that, as for SufI translocation *in vivo*, the *in vitro* translocation of SufI requires an intact membrane potential, physiological temperature, TatABC and a twin-arginine signal motif.

Translocation of SufI *in vivo* requires TatA, TatB and TatC. To test this *in vitro*, IMVs were prepared from wild-type cells overexpressing TatBCE, TatACE or TatABE and from Δ*tatE* mutant cells overexpressing TatABC, and used in translocation reactions. While translocation is detected using TatABCE- and TatABC-overexpression IMVs (Figure 6C, lanes 2 and 3), deletion of either TatA, B or C prevents translocation (lanes 4–6). These data demonstrate that *in vitro* translocation of SufI requires a functional translocase composed of TatABC.

Discussion

We have exploited a novel strategy to detect *in vitro* translocation: first establishing saturation of the translocase *in vivo* and then engineering the overproduction of TatABC, which relieves this saturation. Simultaneous overexpression of SecYEG proportionally enhances *in vitro* Sec translocase activity with chemical amounts of preprotein (Douville *et al*., 1995; Duong and Wickner, 1997), but efficient translocation of radiochemical amounts of Sec substrates such as proOmpA had been seen with wild-type membranes. While the amount of Tat translocation detected in our *in vitro* studies is low, the development of this assay may allow the detection of chaperones or other factors that stimulate translocation.

The development of an *in vitro* import assay for ΔpH/Tat translocation across the chloroplast thylakoid membrane has led to progressive increases in understanding the ΔpH translocation pathway in plants (Mould and Robinson, 1991). Using this assay, several groups have established that translocation across the thylakoid membrane does not require ATP, but is instead dependent upon the membrane ΔpH for both initiation and completion of translocation (Cline *et al*., 1992; Brock *et al*., 1995). The thylakoid ΔpH translocase is capable of translocating tightly folded, misfolded and heterologous folded proteins (Creighton *et al*., 1995; Hynds *et al*., 1998). During substrate translocation, the thylakoid membrane ΔpH is unaffected, suggesting that there is no unregulated flux of protons through this translocase (Teter and Theg, 1998). With the development of an *in vitro* assay for Tat translocation, the ease of bacterial genetics and biochemical analyses make the bacterial Tat translocase an ideal model system for investigations of this translocation mechanism. The similarities between the plant ΔpH and bacterial Tat translocases suggest that their study will be synergistic.

Although prior investigations of *E.coli* Tat translocation have been limited to *in vivo* experiments, these studies revealed some remarkable properties of the Tat translocase. Tat translocase substrates possess N-terminal twin-arginine signal peptides that target preproteins to the translocase, with notable exceptions (Berks, 1996): hydrogenase 2 consists of one large and one small subunit, of which only the small subunit contains a signal peptide bearing a twin-arginine motif. To be translocated by Tat, the large subunit must acquire Ni^{2+} and associate with the small subunit in the cytosol (Rodrigue *et al*., 1999). In the absence of the large subunit, the small subunit is not translocated. In related studies, Santini *et al*. (1998) demonstrated that TorA must first acquire a molybdo cofactor in the cytoplasm to become competent for translocation. The obvious conclusion from these studies is that Tat substrates can only be translocated in folded states. These studies also suggest that the Tat translocase possesses a proofreading activity that can distinguish assembled enzyme complexes from unassembled subunits, and thus proteins with bound cofactors from proteins lacking cofactors (Rodrigue *et al*., 1999). Understanding the mechanisms of translocating folded proteins and the proofreading activity of this translocase should be greatly accelerated by the development of an *in vitro* assay for Tat translocation.

Another intriguing property of the translocase is its capacity to translocate folded proteins, or protein complexes, that display a wide range of molecular weights (~20–142 kDa) (Berks *et al*., 2000a). How the translocase might accommodate such a diverse range of substrates is unclear. It has been proposed that distinct populations of translocase with varying subunit compositions and substrate specificities may exist for this purpose (Chanal *et al*., 1998). Ultimately, purification of the translocase and reconstitution of functional Tat translocase activity will be essential to resolve questions of subunit stoichiometry and substrate specificity.

Materials and methods

Reagents and methods

The β-lactam leader peptidase inhibitor (5S,*6S* penem) was a gift from Karen Dobbs at SmithKlineBeecham Pharmaceuticals (Paetzel *et al*., 1998). Protein was assayed with Bradford (Bio-Rad) and BCA (Pierce) reagents. Anti-mouse and anti-rabbit horseradish peroxidase conjugates and ECL reagent were from Amersham Pharmacia Biotech. Dried gels containing ^{35}S-labeled samples were analyzed using a Molecular Dynamics PhosphorImager and software. For densitometry, autoradiograms were scanned (Lacie, Silverscanner III) and analyzed using IPLab gel software (Signal Analytics).

Bacterial strains and culture conditions

Escherichia coli MC4100 and its Δ*tatA*, Δ*tatB*, Δ*tatC*, Δ*tatE* and Δ*tatA*, *tatE* derivatives have been described previously (Casadaban and Cohen, 1979; Bogsch *et al*., 1998; Sargent *et al*., 1998, 1999). For T7 expression studies, MC4100 and derivatives were converted to λ(DE3) lysogens using a kit (Novagen). For studies using the arabinose-inducible pBAD vectors, arabinose-resistant mutants of MC4100 (ara^r) were isolated on EMB agar with 1% arabinose (Englesberg *et al*., 1962).

Plasmid construction

For regulated expression of TatABCDE, the genes were cloned into pBAD22 under the transcriptional control of an arabinose-inducible promoter. The gene encoding TatE was PCR amplified using primers that incorporated *Xba*I (5′-AGCTCTAGAAGGTATCTATGGGTGAGA) and *Hin*dIII (5′-GTTAAGCTTGGATGGAAGTTAAGTAATCCT) restriction endonuclease cleavage sites and *E.coli* MC4100 chromosomal DNA as a template. The PCR product was gel purified, digested with *Xba*I and *Hin*dIII, and cloned into the corresponding sites of pBAD22, resulting in pTatE. The DNA encoding TatABC and TatABCD was PCR amplified with primers incorporating *Nco*I (5′-CATGACCATGGCCGTGTAACGTATAATGCGGCT) and *Xba*I (5′-CAGCTCTAGAGGCGGTTGAATTTATTCTTC or 5′-AGCTCTAGACTAAAACGCAATCCCAAACAG) restriction sites. The purified PCR products were cloned into the *Nco*I and *Xba*I sites of pBAD22 and pTatE, resulting in pTatABC, pTatABCD,

pTatABCE and pTatABCDE. Expression clones lacking either *tatA* (pTatBCE), *tatB* (pTatACE) or *tatC* (pTatABE) were constructed as outlined above, except that chromosomal DNA from Δ*tatA*, Δ*tatB* or Δ*tatC* strains was used as the template.

For T7-regulated expression and epitope tagging of SufI, the gene was PCR amplified from MC4100 chromosomal DNA with primers incorporating *Nde*I (5′-AGATCATATGTCACTCAGTCGGCGTCAGTTC) and *Xho*I (5′-GTAGCTCGAGCGGTACCGGATTGACCAACAGTTGC) restriction sites and cloned into the respective sites of pET25b (Novagen). The resulting clone (pET-SufI) encodes SufI with C-terminal HSV and His_6 tags. For co-expression of the Tat proteins and SufI, a SufI expression cassette including the T7 promoter and the lacI repressor was PCR amplified with primers (5′-TCTAGAATTCAAAAAACCCCT-CAAGACCCGTTTAGAGG and 5′-TGCTGGATCCAGACATCATAA-GTGCGGCGACGATAG) incorporating *Eco*RI and *Bam*HI restriction sites from pET-SufI, and cloned into the corresponding sites of pSU38 (kanamycin^r), resulting in pSU-SufI (Bartolome *et al*., 1991). The RR to KK derivative of SufI was constructed by PCR using a primer (5′-GTACCATATGTCACTCAGTAAGGAAGCAGTTCATTCAGGC-ATCGGGGATTG) that altered arginine codons 5 and 6 of the SufI signal peptide to lysines.

Antisera

Peptides corresponding to TatA (CQDADFTAKTIADKQAD and CEQAKTEDAKRHDKEQV), TatB (CASLTNLTPELKASMDE and CAEPKTAAPSPSSSDKP), TatC (CMSVEDTQPLITHLIE and CREEENDAEAESEKTEE) and TatE (CDLGAAIKGFKKAMNDD and CKGADVDLQAEKLSHKE) were coupled to keyhole limpet hemocyanin and antisera were generated in New Zealand white rabbits. For affinity purification, peptides were coupled to Sulfolink and antibodies isolated as described previously (Duong and Wickner, 1997). SufI protein was isolated as a His_6-tagged fusion protein using Ni^{2+} affinity chromatography, as described previously (Yahr *et al*., 1996).

Pulse–chase experiments

For pulse–chase experiments, unless stated otherwise, saturated cultures were diluted 1:50 into M9 minimal media with 40 μg/ml each amino acid except methionine and with 1% fructose and the appropriate antibiotics, and shaken at 37°C. At A_{600} = 0.4, aliquots were transferred to microfuge tubes pre-warmed to 37°C and IPTG was added to 1 mM for the indicated times. Cells were pulsed for 1 min with 0.1 μCi/ml ^{35}S Easy Tag EXPRESS labeling mix (NEN Life Sciences Products) and chased with unlabeled methionine (500 μg/ml final). Samples were removed as indicated, mixed with TCA (12.5% final) and incubated on ice for 30 min. Precipitates were collected by centrifugation (12 500 *g* for 12 min at 4°C), suspended in 1 ml cold acetone and sedimented, dried and processed for nickel-nitrilotriacetic acid (Ni-NTA) precipitation as follows. Protein was solubilized in 50 μl of 50 mM Tris–HCl pH 8.0, 1% SDS by heating for 3 min at 95°C. Following the addition of 450 μl of RIPA buffer (50 mM Tris–HCl pH 7.9, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS; Brundage *et al*., 1992), SufI was adsorbed to either 15 μl of packed Ni-NTA–agarose (Qiagen) or anti-SufI protein A–Sepharose beads. Beads were nutated (90 min, 4°C), suspended twice in 1 ml RIPA buffer and sedimented, and proteins were eluted by SDS–PAGE sample buffer and heating to 95°C for 5 min. Samples were analyzed by 12% SDS–PAGE and fluorography.

In vitro translocation

For IMV preparation, strains carrying the Tat expression plasmids were diluted to an A_{600} of 0.05 in Luria–Bertani media containing 1% arabinose. At A_{600} = 1.0, cells were harvested and IMVs were prepared as described previously (Douville *et al*., 1995). SufI substrate was labeled with [^{35}S]methionine using a coupled transciption–translation system (Promega). Following synthesis, the labeling reactions were centrifuged (10 min at 100 000 *g*), resulting in a clarified extract of SufI substrate. Translocation reactions contained 5 μl of 10 × TL buffer (500 mM Tris–HCl pH 8.0, 500 mM KCl, 50 mM $MgCl_2$), 300 μg/ml IMVs, 200 μg/ml lipid-free BSA, 2 mM ATP and 5 mM NADH. Reactions (30 μl) were pre-warmed to 37°C for 3 min prior to the addition of 20 μl of the clarified SufI extract. Following a 60 min incubation at 37°C, reactions were transferred to ice, the volume adjusted to 100 μl with TL buffer and proteinase K was added to 1 mg/ml for 15 min. Membranes were recovered by centrifugation (100 000 *g* for 10 min at 4°C) and suspended in 100 μl of TL buffer. Protein was precipitated by adding 165 μl of 25% TCA, collected by centrifugation, suspended in 1 ml of cold acetone and sedimented, suspended in 40 μl of SDS–PAGE sample buffer and heated for 5 min at 100°C. Samples were analyzed by 12% SDS–PAGE and fluorography.

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