Signal peptide–chaperone interactions on the twin-arginine protein transport pathway

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The twin-arginine transport (Tat) system is a protein-targeting pathway of prokaryotes and chloroplasts. Most *Escherichia coli* Tat substrates are complex metalloenzymes that must be correctly folded and assembled before transport, and a preexport chaperone-mediated “proofreading” process is therefore in operation. The paradigm proofreading chaperone is TorD, which coordinates maturation and export of the key respiratory enzyme trimethylamine *N*-oxide reductase (TorA). It is demonstrated here that purified TorD binds tightly and with exquisite specificity to the TorA twin-arginine signal peptide *in vitro*. It is also reported that the TorD family constitutes a hitherto unexpected class of nucleotide-binding proteins. The affinity of TorD for GTP is enhanced by initial signal peptide binding, and it is proposed that GTP governs signal peptide binding-and-release cycles during Tat proofreading.

ligand binding | molecular chaperone

The twin-arginine transport (Tat) system is a protein-targeting pathway found in the cytoplasmic membranes of many prokaryotes and the thylakoid membranes of chloroplasts (1, 2). Tat-targeted substrate proteins are synthesized as precursors with N-terminal twin-arginine signal peptides that exhibit distinctive SRRxFLK amino acid motifs (3). In *Escherichia coli*, the TatA, TatB, and TatC membrane proteins form the core components of the Tat translocase (1). The TatBC unit is the twin-arginine signal peptide recognition module (4), whereas multiple copies of TatA generate the protein-conducting channel (5–7). Protein translocation is powered solely by the transmembrane proton gradient, and there is some evidence to suggest the Tat translocase acts as a genuine protein–proton antiporter (8).

Most remarkably, the Tat system only transports fully folded proteins (9), and most *E. coli* Tat substrates must bind complex cofactors, fold, and, in some cases, oligomerize before transport can proceed (1). The Tat translocase itself is thought to accept or reject proteins for transport through a quality control activity (9). Although this Tat quality control mechanism would be applied to all proteins presented for export, some complex Tat substrates are subjected to an earlier chaperone-mediated screen that operates to prevent premature targeting of immature proteins before cofactor-loading, correct folding, or docking of partner proteins has occurred. To distinguish from Tat quality control, this important complementary system is termed “Tat proofreading” (10).

The *E. coli* trimethylamine *N*-oxide reductase (TorA) is a Tat-dependent periplasmic redox enzyme that binds a molybdenum cofactor. TorA is the archetypal model Tat substrate, its twin-arginine signal peptide being the most heavily studied and exploited in the field. Loading of the cofactor is an essential prerequisite to TorA transport (11) and the TorD protein was originally identified as a cytoplasmic accessory protein required for efficient cofactor loading into the TorA apoenzyme (12–14). The 3D structure of a TorD homolog from *Shewanella massilia* showed that this family of proteins comprise two separate α -helical domains connected by a short hinge region (15).

Overproduced *S. massilia* TorD was isolated as a mixture of monomers, domain-swapped homodimers, and homotrimers (16), and the crystal structure is of the dimer form (15). TorD was initially not expected to bind the twin-arginine signal peptide directly, however genetic experiments suggested *E. coli* TorD could indeed recognize the TorA signal peptide during the preexport proofreading process (10). Thus, TorD performs dual roles by assisting cofactor insertion into the TorA enzyme (10, 12–14) and facilitating Tat proofreading by recognition of the TorA twin-arginine signal peptide (10).

Twin-arginine signal peptides exhibit common core tripartite structures, including polar n-regions, relatively hydrophobic h-regions, and polar c-regions that usually contain signal peptidase cleavage sites (3). The twin-arginine motif is always located at the boundary between the n- and h-regions, with the arginine pair being critical for successful translocation (1). Definitive physiological functions have not yet been assigned to the n-regions of any Tat signal peptides, although c-regions sometimes contain a positively charged Sec-avoidance motif thought to prevent mistargeting of some Tat substrates (17).

Here, recent advances in understanding the relationship between TorD and the twin-arginine signal peptide in the Tat proofreading process are reported. By using *in vitro* molecular techniques, tight binding of a synthetic TorA twin-arginine signal peptide to purified TorD is demonstrated. Signal peptide binding by TorD is not critically dependent on an intact twin-arginine motif, thus establishing that the preexport Tat proofreading process stands alone from Tat translocation. Finally, it is revealed that TorD is a guanosine nucleotide binding protein and that initial binding of the TorA signal peptide by TorD enhances the chaperone’s affinity for GTP.

Materials and Methods

Plasmid Construction and Protein Methods. *E. coli torD* was amplified by PCR and cloned as an NcoI/BglII fragment into pQE60 (ampicillin-resistant, Qiagen, Valencia, CA), which incorporates a C-terminal hexahistidine tag onto recombinant proteins to give pAAF3. Modified *torD* genes were generated by QuickChange (Stratagene) with pUNI-*torD1* (ampicillin-resistant) (10) or pAAF3 as template. Cloning fidelity was verified by DNA sequencing.

Protein production and purification was as described in ref. 18, and protein concentrations were determined by the Lowry method (19). SDS/PAGE and Western immunoblotting were as described in ref. 10.

The *in vivo* Tat proofreading assay was performed with the reporter strain RJ607 (*torA::hybO*, Δ *hybA*) as described in ref. 10. Briefly, RJ607 harboring TorD-producing plasmids was

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Abbreviations: ITC, isothermal titration calorimetry; Tat, twin-arginine transport.

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cultured anaerobically in 0.5% (vol/vol) glycerol/0.4% fumarate (wt/vol), harvested, resuspended in 20 mM Tris-HCl, pH 7.5, to 0.1 gram of cells per milliliter and assayed for hydrogen::benzyl viologen oxidoreductase activity (10). Custom peptides were synthesized by Alta Bioscience (Birmingham, United Kingdom), Severn Biotech (Kidderminster, U.K.), Sigma-Genosys (The Woodlands, TX), or Mimotopes (Raleigh, NC).

Analytical Ultracentrifugation. Sedimentation-equilibrium experiments were performed in a Beckman Optima XL-I analytical ultracentrifuge equipped with scanning absorbance optics and an An50Ti rotor. TorD proteins were dialysed against 20 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 110 μ l of aliquots placed in charcoal-filled Epon double-sector cells fitted with quartz windows. Reference sectors were filled with dialysis buffer (120 μ l), and centrifugation was performed at 15,000 rpm and 20°C. Data were collected at 280 nm, and equilibrium was confirmed by the absence in any difference between values collated 4 h or more apart. Partial specific volumes for TorD proteins were calculated from the amino acid sequences by using the program SEDNTERP and data were analyzed with the software ULTRASCAN 6.2 and chelated by using ORIGIN 5.0 software.

Isothermal Titration Calorimetry (ITC). ITC was carried out at 28°C by using a VP-ITC titration calorimeter (Microcal, Amherst, MA) and by following standard procedures (20). TorD proteins were dialysed against 20 mM phosphate buffer, pH 7.5, or the same buffer containing 500 mM NaCl and degassed before being loaded into the sample cell (1.4-ml working volume). Peptide ligands were prepared as 1 mM stocks in dialysis buffer and titrated from a 290- μ l syringe into the sample cell stirred at 300 rpm. The heat change for the dilution of peptide in the absence of TorD was recorded for each experiment and subtracted from the measured heat change of ligand binding to protein. Data analysis was performed with the ORIGIN software supplied by Microcal.

Fluorimetry. Measurements of intrinsic tryptophan fluorescence emission were made by using a PerkinElmer LS-55 spectrofluorimeter thermostated at 25°C. Concentrated (50 mg/ml) TorD was dialysed into 20 mM phosphate buffer, pH 7.5, and degassed dialysis buffer was used for all fluorescence experiments. A working TorD concentration of 0.3 μ M and 3-ml quartz cuvettes were used. The excitation wavelength was 295 nm (slit width, 5 nm) and emission spectra were recorded from 310 to 390 nm (slit width, 10 nm). Dissociation constants were calculated by plotting relative fluorescence against ligand concentration and curve-fitting with the Solver add-in available for Microsoft EXCEL software.

Molecular Modeling. A theoretical TorD-GTP binary model was generated by using the available coordinates for *S. massilia* TorD (15). Guanine was manually imposed onto one oxidized DTT molecule by using the program O (21), and an initial model was chosen on the basis of the potential formation of a hydrogen bond between the guanine N^7 atom and the main chain carbonyl oxygen of *S. massilia* TorD E32. Replacing guanine with guanosine triphosphate, the initial model was refined with three rounds of manual model building with the program O alternated with conjugate gradient positional refinement using the program CNS (22).

Results

TorD Binds the TorA Twin-Arginine Signal Peptide *in Vitro*. Genetic evidence suggests that the Tat proofreading activity of TorD depends on direct recognition of the TorA signal peptide (10). To characterize *in vitro* the TorA signal peptide binding activity, *E. coli* TorD was purified. A single species was isolated with a

Fig. 1. TorD monomer contains a single signal peptide binding site. Determination of the native molecular mass of *E. coli* TorD in the absence and presence of a twin-arginine signal peptide by sedimentation equilibrium. Each sample contained 0.4 mg/ml ($\approx 15 \mu$ M) TorD, and sedimentation was monitored at 280 nm in the absence of any ligands, resulting in a 23.6-kDa species (A) and, in the presence of 133 μ M TorA-SP_{10–36} synthetic peptide, a 27.0-kDa species (B). (Lower) The sedimentation data for each species together with simulated single-component sedimentation curves for the molecular masses given. (Upper) Residuals between the experimental data and the fitted curves.

molecular mass determined as 23.6 kDa by analytical ultracentrifugation (Fig. 1A), which compares well with the predicted 23,567 Da for the recombinant protein.

Binding of the TorA signal peptide by TorD was investigated by using ITC. Because of its excessive length, the TorA signal peptide was synthesized in two portions (Table 1). Peptides covering residue N2 through T22, covering the extended n-region and the twin-arginine motif (TorA-SP_{2–22}), and S10 through R36, covering the twin-arginine motif, the h-region, and the Sec-avoidance motif (TorA-SP_{10–36}), were synthesized. Titration of TorD with TorA-SP_{10–36} generated negative enthalpy values and a sigmoidal binding curve that reached a clear saturation point (Fig. 2B), indicating binding of this peptide by TorD. Analysis of the binding isotherm gave a best fit to a noncooperative model with a binding stoichiometry (n) of 0.78 ± 0.02 and a K_D of $\approx 1 \mu$ M for this datum (Fig. 2B). Titration of TorD with the alternative TorA-SP_{2–22} peptide suggested much weaker binding (Fig. 2A). TorD, therefore, binds tightly *in vitro* to the central core structure of the TorA twin-arginine signal peptide over and above the variable n-region.

To gain corroborative evidence of signal peptide binding, TorD was subjected to a sedimentation equilibrium procedure in the presence of excess TorA-SP_{10–36} peptide (Fig. 1B). The mass recorded by following TorD sedimentation was 27.0 kDa (Fig. 1B), an observed mass increase of 3.4 kDa when compared with TorD alone (Fig. 1A) representative of 1.17 times the theoretical mass of the TorA-SP_{10–36} peptide (2.9 kDa). This finding, taken together with the ITC experiment (Fig. 2B), points strongly to a single signal peptide binding site on each TorD monomer.

It is immediately obvious from these data that TorD does not oligomerize into dimer or trimer forms in the presence of signal peptide. Indeed, the physiological significance of the oligomerization exhibited by *S. massilia* TorD (16) remains somewhat ambiguous and is clearly not central to the signal peptide binding process.

Having established the presence of a single peptide binding site on TorD, a one-site, noncooperative, binding model was used to determine the dissociation constants of various synthetic peptides by ITC. This model underscored the tight binding of the TorA-SP_{10–36} peptide by TorD (Table 1), and the specificity of this interaction was tested next. Peptides covering N14 through K34 of the *E. coli* hydrogenase-2 Tat signal peptide (HyO-SP_{14–24}) and N2 through N28 of the TorA signal peptide from *Shewanella oneidensis* (Shew-SP_{2–28}) were synthesized (Table 1). Despite the similar overall physicochemical properties displayed by the alternative peptides, the *E. coli* TorD protein bound only

Table 1. *E. coli* TorD binds specifically to the *E. coli* TorA signal peptide

Protein	Peptide	Peptide sequence	K _D at 0 mM NaCl, μ M	K _D 500 mM NaCl, μ M
TorD	TorA-SP ₂₋₂₂	NNNDLFQASRRFLAQLGGLT	76 $\pm$ 5	192 $\pm$ 31
TorD	TorA-SP ₁₀₋₃₆	SRRFLAQLGGLTVAGMLGPSLLTPRR	1.7 $\pm$ 0.3	1.8 $\pm$ 0.1
TorD	TorA-SP _{KK}	SKKRFLAQLGGLTVAGMLGPSLLTPRR	2.6 $\pm$ 0.1	1.8 $\pm$ 0.1
TorD	TorA-SP ₁₅₋₃₆	LAQLGGLTVAGMLGPSLLTPRR	8.0 $\pm$ 0.4	16 $\pm$ 1
TorD	TorA-SP ₂₃₋₃₆	VAGMLGPSLLTPRR	30 $\pm$ 4	>110
TorD _{R22A}	TorA-SP ₁₀₋₃₆	SRRFLAQLGGLTVAGMLGPSLLTPRR	2.7 $\pm$ 0.2	5.7 $\pm$ 0.6
TorD _{D124A}	TorA-SP ₁₀₋₃₆	SRRFLAQLGGLTVAGMLGPSLLTPRR	5.1 $\pm$ 0.7	5.4 $\pm$ 0.3
TorD _{H125A}	TorA-SP ₁₀₋₃₆	SRRFLAQLGGLTVAGMLGPSLLTPRR	4.8 $\pm$ 0.4	11 $\pm$ 1
TorD	HybO-SP ₁₄₋₃₄	NRRDFMKLAAALAAATMGLSSK	∞	∞
TorD	Shew-SP ₂₋₂₈	NRRDFLKGLASTSFVALGSSVLAPLN	∞	∞

Dissociation constants derived from ITC measurements of peptide binding to native and variant TorD proteins. Titrations were performed at 28°C with 46 μ M protein in 20 mM phosphate buffer, pH 7.5, and 0 or 500 mM NaCl where indicated. Peptides were prepared as 1 mM stocks, and 25 $\times$ 10- μ L injections were made into the reaction vessel to 175 μ M (final concentration). Data were fitted to a one-site, noncooperative binding model with Microcal ORIGIN software. Each value is presented with its corresponding SEM ($n = 3$). Bold, underlined characters are key residues of the twin-arginine motif. Italic typeface indicates residues of the h-region. ∞ , No binding detected by this technique.

to the peptide modeled on *E. coli* TorA (Table 1). These experiments therefore highlight the exquisite specificity of this tandem chaperone/signal peptide system.

Characteristics of Signal Peptide Binding. The signal peptide arginine pair is essential for Tat transport of the TorA precursor (23); however, it is not known whether the twin-arginine motif has a role in the preexport proofreading process. A synthetic TorA signal peptide essentially identical to TorA-SP_{10–36} but

with twin-arginine residues replaced by twin-lysine residues was synthesized (Table 1). The twin-lysine peptide (TorA-SP_{KK}) displayed identical TorD binding characteristics to its twin-arginine counterpart (Table 1). Thus, the twin-arginine motif itself is clearly not the overarching signal recognition factor.

To further dissect the signal peptide recognition activity, the TorA-SP₁₀₋₃₆ peptide was synthesized in two truncated forms. First, the positively charged twin-arginine motif was completely omitted to give peptide TorA-SP₁₅₋₃₆, and, second, a peptide

Fig. 2. TorD binds the core structure of the TorA signal peptide. Calorimetric titration of TorD with synthetic peptides. (Upper) Raw data for the heat effect during titration. (Lower) The binding isotherms. All titrations were performed at 28°C with 46 μ M TorD in 20 mM phosphate buffer, pH 7.5. Injections (25 $\times$ 10 μ l) of peptide ligands (1 mM stocks) were made to the stoichiometric excesses shown. (A) Weak binding of the TorA-SP_{2–22} peptide to TorD. (B) Strong binding of the TorA-SP_{10–36} peptide to TorD. The best fit to the TorA-SP_{10–36} datum gave $n = 0.78 \pm 0.02$ peptide binding sites, $\Delta H = -14.6 \pm 1.0$ kcal/mol (1 cal = 4.18 J), and $\Delta S = -6.6 \pm 0.9$ kcal/mol.

GTP binding affinity in the presence of excess signal peptide (Fig. 3B). This result is consistent with the prediction that R22 could be a GTP ligand and underscores the intimate linkage of the ligand binding events. Taken together, these data point strongly to a ligand-binding mechanism for which the initial interaction with signal peptide results in a repositioning of the R22 side chain, which in turn confers a (physiologically essential) higher affinity for GTP on the TorD Tat proofreading protein.

Discussion

It has been demonstrated here that *E. coli* TorD binds directly to the *E. coli* TorA twin-arginine signal peptide and that the interaction is not dependent on the signal peptide extended n-region. This finding is important because many TorA homologs, including those from *Shewanella*, do not display n-extended twin-arginine signal peptides, despite expressing TorD homologs. The establishment that Tat proofreading chaperones could bind the central cores of Tat signal peptides potentially increases the scope of this type of proofreading mechanism to many other complex Tat substrates.

Once enzyme assembly is complete, it is important that Tat proofreading chaperones rapidly dissociate to allow transport to proceed. It is possible that the mechanism of signal peptide release by TorD follows the principles of other signal peptide or nascent chain binding proteins in that nucleotide-binding steps may govern this process. Ligand binding by TorD appears to be a reciprocal process in which the signal peptide binds first, thus increasing affinity of the chaperone for GTP. The affinity of TorD for GTP is reminiscent of the low-affinity ATP binding site on the SecA protein ($K_D = 340 \mu\text{M}$) that is thought to provide a regulatory role (27). It is indeed conceivable that the GTP binding activity of TorD is regulatory rather than catalytic because we have been unable to detect any intrinsic or peptide-induced GTPase activity associated with the purified protein. However, if GTP hydrolysis is ultimately required, one possibil-

ity is that there are missing components from this highly purified system. Protein-protein interactions mediated by GTP binding proteins conjures images of eukaryotic G protein systems in which guanine nucleotide exchange factors or GTPase-activating proteins are required to activate small G proteins (32). It is tempting to speculate that a degree of continuity might be given to the cellular Tat proofreading process if the known or predicted proofreading chaperones (e.g., TorD, DmsD, HyaE, and HybE) were acting as specialist "adaptors" for a single general activating enzyme.

The signal peptide twin-arginine motif is probably not required for TorD-mediated Tat proofreading, which adds weight to the argument that TorD family proteins are not themselves Tat-targeting factors (33). The twin-arginine motif is primarily a TatC recognition motif and is too highly conserved across enzymes, species, and kingdoms to account for the very specific recognition event between *E. coli* TorD and the *E. coli* TorA signal peptide. However, interaction of the TorD/precursor complex with an additional membrane-bound recognition factor before transport cannot be ruled out. For instance, the TorD homolog DmsD is reportedly attached to the membrane under Tat transport conditions (34), and very recently two uncharacterized *E. coli* membrane proteins have been identified that could be important in Tat targeting (35).

Future challenges will be to generate structural information on the nature of the twin-arginine signal peptide and nucleotide binding sites on TorD and to elucidate the molecular mechanism of controlled signal peptide release by TorD that is ultimately required to complete the Tat proofreading cycle.

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