Abstract The twin-arginine translocation (Tat) system is a bacterial protein targeting pathway. Tat-targeted proteins display signal peptides containing a distinctive SRRxFK ‘twin-arginine’ motif. The Escherichia coli trimethylamine N-oxide reductase (TorA) bears a bifunctional Tat signal peptide, which directs protein export and serves as a binding site for the TorD biosynthetic chaperone. Here, the physical interaction between TorD and the TorA signal peptide was investigated. A single substitution within the TorA signal peptide (L31Q) was sufficient to impair TorD binding. Screening of a random torD mutant library identified a variant TorD protein (Q7L) that displayed increased binding affinity for the TorA signal peptide.

1. Introduction

The Tat (twin-arginine translocation) system is a protein targeting pathway found in many prokaryotes [1]. Tat-targeted proteins are synthesised as precursors with N-terminal signal peptides containing SRRxFK ‘twin-arginine’ motifs [2]. Twin-arginine signal peptides have a tripartite structure including a polar N-terminal region (n-region), a moderately hydrophobic region (h-region), and a C-terminal region (c-region) that often contains a protease cleavage site [2,3]. The twin-arginine motif is always located at the junction between the n- and h-regions [1,2]. Proteins bearing Tat signal peptides are transported by the membrane-bound Tat translocase machinery, the physiological role of which is to move fully folded proteins across the cytoplasmic membrane [1].

Escherichia coli produces 27 Tat-targeted proteins [4]. The majority of these are cofactor-containing enzymes and, in most cases, these must acquire their prosthetic groups in the cytoplasm before export (e.g. [5]). The E. coli trimethylamine N-oxide (TMAO) reductase (TorA) is a Tat-targeted enzyme that binds bis-molybdopterin guanine dinucleotide (MGD) as its single cofactor [6]. Biosynthesis of TorA is governed by TorD, a member of a ‘chaperone’ family dedicated to molybdo-protein assembly [7]. TorD is involved in cofactor loading [8–14] and also performs a ‘Tat proofreading’ function, which prevents premature targeting of TorA until all biosynthetic processes are complete [15]. Tat proofreading involves a direct, specific interaction between TorD and the TorA signal peptide [15,16], however the mechanism of signal peptide recognition and release is not fully understood.

In this work, the molecular basis of the interaction between TorD and the TorA signal peptide has been studied. A leucine-rich region within the signal peptide h-region is shown to be involved in TorD binding both in vivo and in vitro. In addition, a variant, but physiologically active, TorD protein was identified that displays increased affinity for native and variant TorA signal peptides.

2. Materials and methods

2.1. Bacterial strains and plasmids

The two-hybrid reporter strain was E. coli BTH101 (cya) [17]. Plasmid pSH3 [13] encodes a fusion between the TorA signal peptide (codons 1–42) and the T18 fragment of Bordetella pertussis toxin

Abbreviations: Tat, twin-arginine translocation; TMAO, trimethylamine N-oxide
Strains carrying chromosomal mutations in torA were constructed by mutating the torA sequence present in pTor1 [20]. The torA L3IQ allele was moved into pMAK705 [21] and recombined onto the MC4100 chromosome to yield GB430. The ΔtorD allele from FTD100 [15] was introduced into GB430 using the method of Hamilton et al. [21] to give strain GB430-D.

Strain RJ607-M was constructed by moving the ΔmnaA::AprR allele from MC4100mnaA (B. Ize and T. Palmer – unpublished) by P1 transduction into RJ607 [15]. FTD100 (ΔtorD) has been described [15], and GB420 (as MC4100, ΔtorA) was constructed by assembling a ΔtorA allele in pMAK705 before replacement of the native gene on the chromosome as described [21].

Site-directed mutagenesis was by Quikchange (Stratagene) and all clones were sequenced. During genetic manipulations E. coli was grown aerobically in Luria-Bertani (LB) medium [22]. Physiological characterisations were carried out on strains grown anaerobically in LB supplemented with 0.5% (v/v) glycerol and either 0.4% (w/v) TMAO or 0.4% (w/v) fumarate.

2.2. Protein methods

Overproduction and purification of TorDwt and φMalE-TorAwt was as described [19,23]. Subcellular fractions were performed by a lysozyme/EDTA method [24]. TMAO::benzyl viologen (BV) oxidoreductase and Hydrogen::BV oxidoreductase activities were measured as described [15,23]. SDS-PAGE and Western immunoblotting was as described [26,27]. Antisera to the TorA signal peptide (residues 2–16) was generated by Severn Biotech (Kidderminster, UK), while anti-TorA and anti-TorD sera were as described [15,28]. Interactions detected by the bacterial two-hybrid system were quantified by β-galactosidase activity [17] in the BTH101 grown aerobically to mid-log phase at 30 °C in LB medium.

2.3. Isothermal titration calorimetry

ITC was performed in 50 mM Tris·HCl (pH 7.5) at 28 °C in a VP-ITC microcalorimeter (MicroCal Inc.). TorDwt (100 μM) was loaded into the syringe, while the sample cell contained 10 μM φMalE::TorAwt fusion protein. Titrations consisted of 35 × 8 μl injections of TorDwt.

2.4. Molecular modeling of the TorD monomer

Homology modeling techniques were used to construct a three-dimensional model of the E. coli TorD monomer. The structure of TorD from Shewanella massilia [29] was used as a modeling template. This dimeric crystal structure (1NIC) was solved at 2.4 Å resolution and sequence identity between template and model sequence was 32% over 170 aligned residues. To construct a TorD monomer the loops between helix 6 and helix 7, which link the two globular units forming the dimer, and one of the two globular units were deleted. The now missing loop between helix 6 and helix 7 was modeled on the two remaining partial protein chains to form the E. coli TorD monomer. All loops were modeled using the YASARA program (http://www.yasara.org) by searching a non-redundant subset of the protein data bank for loops with similar sequence and start and end anchor points. Subsequently, side-chains were modeled and the model was optimized using the Yamber2 force field [30]. A coordinate file of the model is available from the authors upon request.

3. Results

3.1. Glutamine-scanning mutagenesis of the hydrophobic region of the TorA signal peptide

An initial study of TorA signal peptide binding by TorD indicated that recognition involved the hydrophobic h-region [16]. In order to rapidly assess the roles of TorA h-region side-chains in TorD binding, a program of mutagenesis using a bacterial two-hybrid system was undertaken. Cristóbal et al. [3] demonstrated that glutamine occurred rarely in Tat signal peptide h-regions, thus TorA residues L18 through L32 were substituted individually with glutamine and the TorD interaction observed (Fig. 1A).
Mutations were introduced into plasmid pSig3 [15], which when co-expressed with pT25-TorD in the E. coli cya strain BTH101 allows the detection of the TorA–TorD interaction in vivo (Fig. 1B). Substitutions of TorA G19, G20, V23, G28, L27, L31 and L32 with glutamine resulted in low levels of $\beta$-galactosidase activity (Fig. 1B). Indeed, the L31Q and L32Q variants of the TorA signal peptide displayed essentially undetectable levels of interaction with TorD (Fig. 1B). Thus regions of the TorA signal peptide important in binding, in particular a leucine-rich segment at the C-terminus including L27, G28, L31 and L32, were identified (Fig. 1B).

### 3.2. The L31Q variant of the TorA signal peptide is impaired in TorD binding

Isothermal Titration Calorimetry (ITC) [16,19] was used to compare and contrast the apparent dissociation constants ($K_d$s) for the interaction between TorD and the TorA L31Q variant signal peptide. Titration of the native TorA signal peptide with purified TorD resulted in a sigmoidal binding curve that reached a clear saturation point (Fig. 2A) resulting in an apparent $K_d$ of 59 nM (Table 1). Titration of TorD against a MalE-TorA fusion protein bearing the L31Q substitution, which showed a negligible interaction with TorD in vivo (Fig. 1B), suggests these proteins can still interact in vitro (Fig. 2B). However, the best fit to the L31Q TorA–TorD data (Fig. 2B) gives an apparent $K_d$ of 1147 nM (Table 2).

### 3.3. The L31Q variant of the TorA signal peptide is transport active

To test the physiological effect of the TorA L31Q substitution on the biosynthesis of the native enzyme, a strain (GB430) was constructed that would produce a modified TorA derivative of TorD. Mutations were introduced into plasmid pSig3 [15], which encoded a variant TorD ($\alpha$-TorD or 'Q7L') and pUNI-Q7L ($\alpha$-Q7L) were cultured anaerobically in LB medium supplemented with 0.5% (v/v) glycerol and 0.4% (w/v) TMAO before being fractionated into periplasm (p, black bars), total membranes (m, grey bars) and cytoplasmic (c, white bars) fractions. TMAO:BV oxidoreductase activity was then determined. Activity is expressed as $\mu$mol of benzyl viologen oxidized per minute per gram of cells. The error bars represent S.E.M. ($n = 3$).

### 3.4. Isolation of TorD variants that show enhanced recognition for TorA L31Q

A genetic screen was undertaken to isolate TorD proteins that could interact with the L31Q TorA variant signal peptide. The screen was based on the bacterial two-hybrid system and strain BTH101 was co-transformed with the plasmid encoding TorA L31Q-T18 and a random library of torD mutants in pT25. Positive interactors were detected on MacConkey maltose indicator plates (where red colonies indicate an interaction), and ultimately two torD clones from an estimated 60000 transformants were isolated that consistently produced red colonies in this test. The two positive 'suppressor' clones were designated 'Suppl', which encoded a variant TorD.

![Fig. 3. Physiological activity of the TorA L31Q and TorD Q7L variants.](image-url)

**Table 1**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>TorD</th>
<th>$K_d$ (nM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native TorA</td>
<td>Native TorD</td>
<td>59 ± 5</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>Native TorA</td>
<td>Q7L TorD</td>
<td>21 ± 1</td>
<td>–0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Native TorA</td>
<td>F180L TorD</td>
<td>177 ± 9</td>
<td>0.7</td>
<td>3</td>
</tr>
<tr>
<td>Native TorA</td>
<td>L189Q TorD</td>
<td>163 ± 10</td>
<td>0.6</td>
<td>2.8</td>
</tr>
</tbody>
</table>

$G = RT \ln(K_d) |

**Table 2**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>TorD</th>
<th>$K_d$ (nM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>L31Q TorA</td>
<td>Native TorD</td>
<td>1147 ± 172</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>L31Q TorA</td>
<td>Q7L TorD</td>
<td>467 ± 20</td>
<td>–0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>L31Q TorA</td>
<td>F180L TorD</td>
<td>765 ± 52</td>
<td>–0.2</td>
<td>0.7</td>
</tr>
<tr>
<td>L31Q TorA</td>
<td>L189Q TorD</td>
<td>882 ± 54</td>
<td>–0.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$G = RT \ln(K_d)$
bearing the three amino acid changes Q7L, F180L and L189Q, and ‘Supp2’, which encoded a TorD protein carrying a single Q186L substitution.

3.5. A Q7L substitution in TorD increases its affinity for signal peptides

\[ \text{Beta-galactosidase assays suggested} '\text{Suppl'} \text{ could maintain a more significant interaction with the TorA L31Q signal peptide than 'Supp2' in the in vivo two-hit system (Fig. 4A). Moreover, beta-galactosidase activities suggested tighter interactions were possible between both 'Suppl' and 'Supp2' and the native TorA signal peptide than observed for the native TorD protein (Fig. 4B).} \]

Next, the four ‘Suppl’ and ‘Supp2’ point mutations were separately incorporated into an overexpression vector pQI-TorD. The variant TorD protein encoded by ‘Supp2’ (Q186L) proved recalcitrant to purification and further characterization was not possible. The TorD variants Q7L, F180L, and L189Q, however, were tested for their ability to interact with the TorA signal peptide in vitro. ITC demonstrated that the F180L and L189Q variants showed slight increases in affinity for the TorA L31Q signal peptide relative to native TorD (Table 2). The Q7L TorD variant, however, exhibited a significant increase in affinity for the TorA L31Q signal peptide (Table 2). While the F180L and L189Q TorD variants were found to bind less tightly than native TorD to the native TorA signal peptide (Table 1), the Q7L TorD variant again exhibited a notably higher affinity for the native TorA signal peptide (Table 1). Thus the TorD Q7L substitution generates a protein that binds more tightly in general to signal peptides, rather than compensating directly for the L31Q substitution in the variant signal peptide.

3.6. The TorD Q7L variant is physiologically active

The physiological activity of the variant TorD Q7L protein was assessed. The ability of TorD Q7L to complement a ΔtorD strain was examined and the variant protein indeed retained its biological activity and was able to rescue TMAO reductase levels to normal levels (Fig. 3A). Because of the increased binding affinity for TorA exhibited by the TorD Q7L variant, it was possible that a portion of tightly-bound chaperone could be exported to the periplasm in tandem with the TMAO reductase. Western analysis demonstrated, however, that neither the native TorD protein nor the Q7L variant was exported to the periplasm under any conditions tested (Fig. 3B).

An E. coli reporter strain has been described that allows the assay of the Tat proofreading activity of TorD [15]. The RJ607 strain expresses a chimeric hydrogenase enzyme bearing the TorA signal peptide and displays a low intrinsic hydrogenase activity that can be boosted through co-expression of an active torD gene [15]. Use of this assay demonstrated that the TorD Q7L variant retained its Tat proofreading activity in vivo (Fig. 3C).

4. Discussion

In this work, single side-chain modifications of the TorA signal peptide h-region are shown to be sufficient to disrupt binding by TorD. Combined genetic and biophysical approaches point to a leucine-rich hotspot for TorD binding comprising L27, G28, L31, and L32. As with recent examples of tight protein–protein interactions [31], it is possible that the critical hydrophobic leucine residues of TorA identified here may become buried upon binding by TorD. Indeed, the ITC experiments point to a difference in free energy change (∆AG = RT ln(κvariant/κnative)) of +1.8 kcal/mol for TorD binding to the native versus the L31Q variant TorA signal peptide. This is caused by a combination of a decrease in enthalpy (∆H) of binding (−2.1 kcal/mol), indicative of an increase in hydrogen bonding, together with a decrease in entropy (T∆S) of binding (−4.0 kcal/mol), perhaps indicating a reduced ‘hydrophobic effect. While the structure of the TorA signal peptide h-region in free or TorD-bound form is unknown, helical wheel projections of the h-region suggest a broad TorD-interacting face if plotted as a canonical α-helix (Fig. 5A), or a neater interacting face if plotted as a coiled-coil (Fig. 5B).

TorD Q7L is capable of recognizing a signal peptide bearing the L31Q change. The Q7 residue is conserved across the TorD family and interchangeable with asparagine or aspartate in nature [29,32–34]. Available 3D structures of TorD family proteins show an all-helical fold arranged into 2 domains connected by a linker [29]. Sh. massilia TorD forms a homodimer where the N-terminal domain of one protomer packs onto the C-terminal domain of another (Fig. 5C and ref. [29,33,34]). However, all other TorD structures are monomeric and the signal peptide binding studies of E. coli TorD are also performed on the monomer. Thus a 3D model of the E. coli TorD monomer was built (Fig. 5D and E). The E. coli TorD Q7 residue (equivalent to N7 of the Sh. massilia TorD) is located at the beginning of the first α-helix (Fig. 5E). Helix 1 is important in forming the ‘helix bundle core’ [29] and makes extensive contacts with helix 10 [29]. Indeed, the other amino acid substitutions that had some influence on peptide binding efficacy are located on helix 10 (Fig. 5E). It is possible, therefore, that the Q7L substitution is modifying the interaction between the TorD domains and thus having a long-range effect on the nature of the signal peptide binding site. Alterna-
In conclusion, this work has identified key structural features of a Tat signal peptide required for its recognition and binding by a Tat proofreading chaperone. These features appear to be distinct from those involved in Tat targeting and suggests these two processes may not be mutually exclusive. In addition, a variant TorD chaperone has been identified with an enhanced affinity for its peptide ligand. Both of these findings will facilitate future studies into the structure and function of the paradigmatic TorD to TorA signal peptide interaction. The next issues to address are whether there is an induced secondary structure imposed on the TorA signal peptide upon TorD binding, and exactly where on the TorD surface the key TorA residues are binding.

Acknowledgements: We thank Ms. V. Lyall (UEA, Norwich) for excellent technical assistance, Dr. B. Ize and Dr. P.A. Lee (John Innes Centre, Norwich) for help with bacterial strain construction and random mutagenesis, and Prof. R.J. Turner (Calgary) for helpful suggestions. This work was funded in the UK by the BBSRC through award BBS/B00780/1 (to FS and DJR). T.P. is an MRC Senior Non-Clinical Research Fellow.

References


Fig. 5. Models of the TorA signal peptide and the TorD monomer. (A) Helical wheel projection of the TorA h-region as a canonical α-helix (3.6 residues per turn). (B) Helical wheel projection of the TorA h-region with 3.9 residues per turn. Residues implicated in TorD binding by in vivo and in vitro interaction analysis are colored red. The projections were drawn using the on-line tool available from the University of California, Riverside, at http://rzlab.ucr.edu/scripts/wheel/wheel.cgi. (C) Topology of the domain-swapped TorD dimer [29]. Helices are drawn as rectangles and are numbered sequentially for each of the 2 polypeptide chains (colored red and turquoise). The secondary structure elements that together form a globular domain are indicated using the grey boxes and the loops joining the two globular domains are indicated with dashed lines. (D) Topology of the modeled E. coli TorD monomer. A new loop, indicated in purple, is modeled between helix 6 and helix 7 to create a complete monomeric TorD model. (E) A structure model of the E. coli TorD monomer. The N-terminal helix 1 is shown in cyan and the C-terminal helix 10 in yellow. The loop between helices 6 and 7 is shown in pink. Residue Q7 is highlighted in orange, F180 in blue, and L189 in purple. The ribbon diagram was drawn using PyMol.


