Kinetic Basis for the Competitive Recruitment of TolB by the Intrinsically Disordered Translocation Domain of Colicin E9

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Received 18 November 2011; received in revised form 11 January 2012; accepted 24 January 2012

Available online 30 January 2012

Edited by C. R. Matthews

Keywords: periplasm; Tol–Pal complex; colicin translocation; intrinsically disordered protein

TolB and Pal are members of the Tol–Pal system that spans the cell envelope of Gram-negative bacteria and contributes to the stability and integrity of the bacterial outer membrane (OM). Lipoylated Pal is tethered to the OM and binds the β-propeller domain of periplasmic TolB, which, as recent evidence suggests, disengages TolB from its interaction with other components of the Tol system in the inner membrane. Antibacterial nuclease colicins such as colicin E9 (ColE9) also bind the β-propeller domain of TolB in order to catalyze their translocation across the bacterial OM. In contrast to Pal, however, colicin binding to TolB promotes its interaction with other components of the Tol system. Here, through a series of pre-steady-state kinetic experiments utilizing fluorescence resonance energy transfer pairs within the individual protein–protein complexes, we establish the kinetic basis for such ‘competitive recruitment’ by the TolB-binding epitope (TBE) of ColE9. Surprisingly, the 16-residue disordered ColE9 TBE associates more rapidly with TolB than Pal, a folded 13-kDa protein. Moreover, we demonstrate that calcium ions, which bind within the confines of the TolB β-propeller domain tunnel and are known to increase the affinity of the TolB–ColE9 complex, do not exert their influence through long-range electrostatic effects, as had been predicted, but through short-range effects that slow the dissociation rate of ColE9 TBE from its complex with TolB. Our study demonstrates that an intrinsically disordered protein undergoing binding-induced folding can compete effectively with a globular protein for a common target by associating more rapidly than the globular protein.

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Introduction

Intrinsically disordered proteins (IDPs) play critical roles in a broad spectrum of cell processes in prokaryotes and eukaryotes and are implicated in pathogenesis and disease.1,2 The biological activity of IDPs stems principally from their ability to bind other macromolecules; such folding-upon-binding secures high-specificity but low-affinity complexes, while the plasticity of the unfolded structure allows them to be tailored to different partners.3 The placement of multiple linear binding epitopes within IDPs also predisposes them to being protein–protein interaction ‘hubs’ in cellular networks.4
A major focus of research in this rapidly developing field is determining the molecular mechanisms by which IDPs bind specific protein targets. An emerging theme in IDP biology is how they are able to subvert or modify cellular processes by direct competition with other proteins for binding partners. For example, the disordered CR1 region of the adenoviral E1a oncoprotein competes directly with the disordered transactivation domain of p53 for binding to the structured TAZ2 domain of the general transcriptional coactivator CBP as part of the cell transformation process. The present work focuses on another recently discovered example where an IDP competes with a cellular protein for binding to a target but, in this instance, the competitor is a much larger folded protein where the bound IDP mimics many of its interactions.

The Tol–Pal system spans the cell envelope of Gram-negative bacteria and consists of five proteins. TolA, TolQ, and TolR form an inner membrane (IM) complex, with TolA traversing the periplasmic space. TolB, a 44-kDa soluble periplasmic protein, consists of two-domains: a C-terminal six-bladed β-propeller domain and an N-terminal α/β domain (Fig. 1). The endogenous binding partner of TolB in the Escherichia coli periplasm is the outer membrane (OM) lipoprotein Pal (peptidoglycan-associated lipoprotein, 13 kDa), which binds either to the peptidoglycan or to the C-terminal domain of TolB, with the two interactions being mutually exclusive of each other. The Tol–Pal assembly is coupled to the proton motive force across the IM, and this coupling is required for its primary function of maintaining OM stability, which is most likely expressed at cell division sites to which the assembly is recruited. TolAQR genes deletion of tolAQR genes leads to cell chaining, blebbing of the OM, leakage of periplasmic components to the environment, increased antibiotic and detergent sensitivity, and resistance to colicins and bacteriophages. The protein antibiotic colicin E9 (ColE9) hijacks the Tol–Pal system in the cell envelope of E. coli in order to translocate a single cytotoxic endonuclease (DNase) domain across the OM. A member of the nuclease E group colicins, ColE9, kills E. coli cells by randomly degrading the bacterial genome (Fig. 1). Cell entry is initiated by the high-affinity binding of the colicin to the OM vitamin B12 receptor BtuB. Subsequently, ColE9 recruits a single trimeric porin (OmpF or OmpC) as an OM translocator through which it inserts a large proportion of its N-terminal 83-residue intrinsically unstructured translocation domain (IUTD) via a process called directed epitope delivery. Embedded within the IUTD is the 16-residue intrinsically disordered TolB-binding epitope (TBE) (ColE9 TBE, residues 32–47), which binds in a canyon in the TolB β-propeller domain, overlapping the binding site of Pal. Bonsor et al. showed recently that the N-terminal 12 amino acids

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**Fig. 1.** Formation of the ColE9 translocon complex at the E. coli OM establishes competitive recruitment between colicin and Pal. When the OM-tethered Pal (Protein Data Bank ID: 2HQS) is not associated with peptidoglycan, it is free to bind to the canyon of the β-propeller domain of TolB (Protein Data Bank ID: 2W8B). Following the association of ColE9 with the vitamin B12 receptor BtuB (the structure of ColE3 bound to BtuB is shown; Protein Data Bank IDs 1UJW and 1JCH) through its coiled-coil R-domain, the colicin associates with the OM porin OmpF (Protein Data Bank ID: 2OMF) and utilizes the pore to extend the intrinsically disordered ColE9 TBE to the periplasm, where it must compete with Pal for binding to TolB.
of TolB interact with the IM protein TolA and that this region undergoes a disorder–order transition, which is promoted by ColE9 TBE binding but counteracted by Pal.28 Contact between TolB and TolA is essential for Tol–Pal function in vivo and cellular invasion by the ColE9 endonuclease.28

The IUTD of ColE9 TBE has been shown by NMR spectroscopy to be largely unstructured in solution.29 Upon binding to TolB, the ColE9 TBE mimics several of the interactions made by Pal with TolB, including a water-mediated hydrogen-bond network, but also forms distinct interactions that explain its ability to elicit a different allosteric response from TolB.25,28 An additional factor in the binding of allosteric effectors to the TolB binding site canyon is the tunnel running through the β-propeller domain that accommodates two calcium ions. The bound divalent cations switch the local surface electrostatics at the opening of the β-propeller tunnel (which constitutes the ColE9 TBE/Pal binding site) from predominantly negative to positive, resulting in the equilibrium dissociation

Fig. 2 (legend on next page)
Table 1. Comparing the thermodynamic parameters for the WT TolB–Pal and TolB–TBEp complexes with their fluorescently labeled counterparts used in this study

<table>
<thead>
<tr>
<th></th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$\Delta S$ (kcal mol$^{-1}$)</th>
<th>$K_a$ (μM)</th>
<th>$n$</th>
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<tr>
<td><strong>WT Pal versus WT TolB</strong></td>
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<tr>
<td>–</td>
<td>$-17.7 \pm 0.04$</td>
<td>$-7.9 \pm 0.1$</td>
<td>$0.11 \pm 0.02$</td>
<td>$0.88 \pm 0.009$</td>
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<tr>
<td>+</td>
<td>$-14.8 \pm 0.01$</td>
<td>$-5.2 \pm 0.1$</td>
<td>$0.18 \pm 0.02$</td>
<td>$0.95 \pm 0.01$</td>
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<tr>
<td><strong>FAM–Pal versus TMR–TolB</strong></td>
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<tr>
<td>–</td>
<td>$-15.8 \pm 0.8$</td>
<td>$-5.7 \pm 0.9$</td>
<td>$0.08 \pm 0.01$</td>
<td>$0.86 \pm 0.07$</td>
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<tr>
<td>+</td>
<td>$-10.1 \pm 0.1$</td>
<td>$-0.6 \pm 0.1$</td>
<td>$0.19 \pm 0.02$</td>
<td>$0.94 \pm 0.006$</td>
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<tr>
<td><strong>WT ColE9 (T) versus WT TolB</strong></td>
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<tr>
<td>–</td>
<td>$-23.8 \pm 0.5$</td>
<td>$-16.2 \pm 0.3$</td>
<td>$5.1 \pm 0.34$</td>
<td>$0.81 \pm 0.01$</td>
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<tr>
<td>+</td>
<td>$-27.7 \pm 2.2$</td>
<td>$-18.6 \pm 1.5$</td>
<td>$0.7 \pm 0.2$</td>
<td>$0.7 \pm 0.003$</td>
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<td><strong>TMR–TBE versus FAM–TolB</strong></td>
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</tr>
<tr>
<td>–</td>
<td>$-14.3 \pm 0.8$</td>
<td>$-6.4 \pm 0.3$</td>
<td>$3.02 \pm 0.5$</td>
<td>$0.94 \pm 0.04$</td>
</tr>
<tr>
<td>+</td>
<td>$-20.5 \pm 0.2$</td>
<td>$-12.0 \pm 0.2$</td>
<td>$1.08 \pm 0.08$</td>
<td>$0.98 \pm 0.008$</td>
</tr>
</tbody>
</table>

The WT ColE9 T-domain binds to TolB with identical affinity and thermodynamic parameters as the unlabeled ColE9 TBEp at 20 °C. ITC titrations were performed in 50 mM Hepes–NaOH, 50 mM NaCl, and 1 mM CaCl$_2$ or 10 mM EDTA (pH 7.5) at 37 °C.

Results and Discussion

FRET pairs reporting TolB–Pal and TolB–ColE9 TBE complex formation do not affect binding

In order to shed light on the underlying kinetics of competitive TolB recruitment, we measured the individual association and dissociation rates of the TolB–Pal and TolB–TBE complexes at the physiological temperature of 37 °C. For this purpose, FRET pairs were constructed between Q280C-TolB and S139C-Pal (lacking its N-terminal lipoyl tether) using the maleimide derivatives of fluorescein [donor: fluorescein-5-maleimide diacetate (FAM)] and rhodamine [acceptor: tetramethylrhodamine-6-maleimide (TMR)], respectively, while a synthetic [5-(and-6)-carboxytetramethylrhodamine (TAMRA)] rhodamine-labeled peptide corresponding to the ColE9 TBE (TBEp) was used in combination with fluorescein-labeled TolB (Fig. 2) (see Materials and Methods for details). The donor (fluorescein) was excited at 480 nm, and the resulting FRET was observed either as the quench of the donor emission (fluorescein; emission $\lambda_{max}$ = 515 nm) in the association between FAM–Pal and TMR–TolB or as the increase in the acceptor emission (rhodamine; emission $\lambda_{max}$ = 575 nm) in the association between FAM–TolB and TBEp (TAMRA labeled; Fig. 2). The equilibrium dissociation constants of all protein pairs were directly comparable to those of their unlabeled counterparts, demonstrating that the incorporated fluorophores did not interfere with binding (Fig. 2 and Table 1). The effect of Ca$^{2+}$ on the affinities of both complexes at 37 °C was found to be the same as the trend observed previously at 20 °C, although the absolute values differ. Calcium ions increase the TolB–Pal constants ($K_a$) for the two complexes becoming essentially equivalent at 20 °C (~0.1 μM). In the absence of Ca$^{2+}$, Pal binds significantly more tightly to TolB than the negatively charged ColE9 TBE. Here we address the kinetic basis for the competitive recruitment of TolB from the TolB–Pal complex by the intrinsically disordered ColE9 TBE and the basis for the effect of Ca$^{2+}$ using stopped-flow fluorescence resonance energy transfer (FRET).

![Fig. 2. FRET pairs that monitor TolB–Pal and TolB–ColE9 TBEp complex formation do not affect binding affinity. (a) Fluorescence emission spectra of 37 mM FAM–Pal in the absence (continuous line) and in the presence (dotted line) of 360 mM TMR–TolB. (b) Spectra of 780 mM FAM–TolB in the absence (continuous line) and in the presence (dotted line) of 28 μM TBEp. The excitation wavelength used was 480 nm in both cases, and the bandwidth was set to 3 nm. Spectra were collected in 50 mM Hepes–NaOH, 50 mM NaCl, and 1 mM CaCl$_2$ (pH 7.5) at 37 °C. Insets show the positions of the fluorescent labels in the TolB–Pal complex (a) and the TolB–TBEp complex (b). In (a), Cys280 of TolB (blue) is TMR labeled (red; acceptor), while Cys139 of Pal (green) is FAM labeled (yellow; donor). The same color scheme is used throughout this work. The distance between the two Cys residues is ~29 Å, which is well below the Förster radius for the fluorescein–rhodamine FRET pair at 55 Å. In (b), Cys280 of TolB (blue) is FAM labeled (yellow; donor), while the ColE9 TBEp (green) is TAMRA labeled (red; acceptor) at the terminal Lys of the G48-K54 extension (black). The distance between TBEp C47 and C280 of TolB is ~36 Å but—with the addition of seven extra residues up to Lys54—extends this distance to ~57 Å matching the Förster radius. Representative ITC data for the titration of 25 μM TMR–TolB versus 250 μM FAM–Pal (c) and 25 μM FAM–TolB versus 233 μM TAMRA–TBEp (d) in 50 mM Hepes–NaOH, 50 mM NaCl, and 1 mM CaCl$_2$ (pH 7.5) at 37 °C. The titrations of 250 μM FAM–Pal and 233 μM TAMRA–TBEp into buffer (offset by 0.1 μcal s$^{-1}$; top) were used to correct the data for the heats of dilution. Data were subsequently fitted to a single-site model using Origin (Microcal). See Table 1 for thermodynamic parameters. ITC data therefore indicate that the incorporation of fluorophores does not interfere with the thermodynamics of the TolB–Pal complex or the TolB–TBEp complex.]
$K_d$ compared to the metal-free state, while the opposite is the case for the TolB–TBEp complex (Table 1).

**ColE9 TBE associates with TolB through a simple bimolecular mechanism**

The association between TBEp and TolB was followed by monitoring the increase in TBEp–rhodamine emission (acceptor) fluorescence through a 570-nm cutoff filter. Association kinetics were measured under pseudo-first-order conditions in which one protein was held at 2 μM against at least a 5-fold excess of its counterpart. In the presence of 1 mM Ca$^{2+}$, monophasic profiles were obtained with either protein in excess (Fig. 3a); in each case, no additional events occurred during the dead time of the experiment. Fitting to a single-exponential equation yielded an observed rate ($k_{obs}$) that was dependent on the concentration of the protein in excess (Fig. 4a and b). The slope of $k_{obs}$ versus excess [protein] plots yielded bimolecular association rates ($k_{on}$) of $2.2 \times 10^5$ M$^{-1}$ s$^{-1}$ (TolB in excess) and $1.6 \times 10^5$ M$^{-1}$ s$^{-1}$ (TBEp in excess; Table 2). These values are comparable to other IDPs that bind globular proteins (such as p27 binding CREB, and IA3 binding YPrA), albeit an order of magnitude slower. 6,8 Agreement between the rates obtained by varying the concentration of either TolB or the TolB-binding epitope within the T-

![Fig. 3. Stopped-flow FRET traces following the association and dissociation of the ColE9 TBEp–TolB and TolB–Pal complexes.](image)
The dissociation rate for the TolB–TBEp complex ($k_{off}$) was estimated indirectly from the intercept of the $k_{obs}$ plot when TBEp was in excess (0.5 s$^{-1}$; Table 2) and directly through competition experiments in which a preformed complex of the labeled TolB–TBEp was dissociated by an excess of unlabeled ColE9 (Fig. 3b), with the two values showing close agreement (0.2 s$^{-1}$; Table 2). The macroscopic equilibrium dissociation constants for the binding reaction calculated from the individual rate constants (Scheme 1; $K_d = k_{off}/k_{on}$) were 0.9 μM and 3.1 μM (TolB and TBEp in excess, respectively), which are in good agreement with the value of 1.1 μM determined by isothermal titration calorimetry (ITC) (Table 2), further demonstrating that, under the conditions employed in this study, the ColE9 TBEp binds TolB via a single kinetically discernible step.

Ca$^{2+}$ decreases the dissociation rate of the TolB–ColE9 TBE complex

In order to investigate the effect of Ca$^{2+}$ on TolB–TBEp complex formation, we also performed stopped-flow experiments in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA) (ITC experiments using Chelex-100-treated buffers indicated that TolB stripped of divalent cations bound the ColE9 TBEp identically in the presence and in the absence of 10 mM EDTA; data not shown). We obtained $k_{on}$ values of 1.9×10$^5$ M$^{-1}$ s$^{-1}$ and 1.6×10$^5$ M$^{-1}$ s$^{-1}$ (TolB and TBEp in excess, respectively), which are essentially identical with those obtained in the presence of metal ions (Table 2). Since the association rate of the TolB–TBEp complex is unaffected by Ca$^{2+}$, it appears that binding of the intrinsically unstructured TBE to TolB is not influenced by long-range electrostatic effects, which is surprising given the change in surface electrostatics close to where the metal ions are located in the TolB β-propeller tunnel.27 However, consistent with the absence of such long-range electrostatic effects, we found that the association rate of the TolB–TBEp complex is similarly unaffected by increasing ionic strength (data not shown). However, the $k_{off}$ value in the presence of EDTA increases by 3-fold compared to that in the presence of Ca$^{2+}$ (0.7 s$^{-1}$ and 1.4 s$^{-1}$ for TolB and TBEp in
excess, respectively; Table 2). The overall macroscopic dissociation constants determined in the absence of Ca\textsuperscript{2+} were 3.7 μM and 8.7 μM (Table 2), which are in reasonable agreement with the ITC value of 3.0 μM. It thus appears that the change in surface electrostatics within the TolB binding site exerts only a localized effect on IDP binding, increasing the stability of the complex by decreasing the rate of complex dissociation. This localized effect is most likely a result of Ca\textsuperscript{2+} only influencing the electrostatic potential at the opening of the β-propeller tunnel.\textsuperscript{27} We speculate that the resulting improved binding of the ColE9 TBE may be due either to improved electrostatic complementarity of the TolB–ColE9 complex (TBE has a net negative charge) or to stronger hydrogen bonding by the ColE9 TBE residue Glu42, which engages in a hydrogen-bond network at the mouth of the β-propeller tunnel.\textsuperscript{25}

**Pal associates more slowly with TolB than ColE9 TBE**

As is the case for the association kinetics of the TolB–TBEp complex, only a single phase was observed for the association between TolB and Pal, both in the presence and in the absence of Ca\textsuperscript{2+} (Fig. 3c). Association was followed by monitoring the quench of the fluorescein emission (donor) of labeled Pal through a 495-nm cutoff filter, with FAM–Pal at 1 μM and at least a 5-fold excess of TMR–TolB. The measured bimolecular association rates were 5.4 × 10\textsuperscript{4} M\textsuperscript{-1} s\textsuperscript{-1} (+Ca\textsuperscript{2+}) and 7.6 × 10\textsuperscript{4} M\textsuperscript{-1} s\textsuperscript{-1} (–Ca\textsuperscript{2+}) (Fig. 4c and Table 2). Association experiments in which TMR–TolB concentrations were limiting and FAM–Pal was in excess were unsuccessful, as no significant donor quenching (using a 495-nm cutoff filter) or acceptor emission (using a 550-nm cutoff filter) could be detected (data not shown).

**Table 2.** TolB–Pal and TolB–TBE complex formation pre-equilibrium kinetic parameters and binding constants

<table>
<thead>
<tr>
<th>Protein complex</th>
<th>Excess protein</th>
<th>Ca\textsuperscript{2+}</th>
<th>Rate constants</th>
<th>Binding constants (μM)</th>
<th>Kinetics</th>
<th>ITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TolB–Pal</td>
<td>TolB</td>
<td>+</td>
<td>5.4 (±0.6) × 10\textsuperscript{4}</td>
<td>0.006 (±5 × 10\textsuperscript{-5})</td>
<td>0.11</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>TolB</td>
<td>–</td>
<td>7.6 (±0.9) × 10\textsuperscript{4}</td>
<td>0.004 (±1 × 10\textsuperscript{-5})</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>TolB–TBEp</td>
<td>TolB</td>
<td>+</td>
<td>2.2 (±0.1) × 10\textsuperscript{5}</td>
<td>0.2 (±0.01)</td>
<td>0.9</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>TBEp</td>
<td>+</td>
<td>1.6 (±0.04) × 10\textsuperscript{5}</td>
<td>0.5 (±0.1)</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TolB</td>
<td>–</td>
<td>1.9 (±0.1) × 10\textsuperscript{5}</td>
<td>0.7 (±0.2)</td>
<td>3.7</td>
<td>3.02</td>
</tr>
<tr>
<td></td>
<td>TBEp</td>
<td>–</td>
<td>1.6 (±0.1) × 10\textsuperscript{5}</td>
<td>1.4 (±0.2)</td>
<td>8.7</td>
<td></td>
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</table>

All kinetic traces were obtained in 50 mM Hepes–NaOH, 50 mM NaCl, and 1 mM CaCl\textsubscript{2} or 10 mM EDTA (pH 7.5) at 37 °C. The K\textsubscript{d} estimated from kinetics equals k\textsubscript{off}/k\textsubscript{on}. Standard errors from the linear least-squares regression analysis of k\textsubscript{obs} versus excess [protein] or from the averaging of at least three measurements are shown in parentheses.

\* k\textsubscript{off} values estimated from the single-exponential fit of dissociation experiments.

\*\* k\textsubscript{off} values estimated from the intercept of the pseudo-first-order plots.

A feature of Pal and the ColE9 TBE binding TolB is the relative simplicity of their association profiles, belying the extensive conformational changes occurring in the two complexes. In the case of the ColE9 TBE, the intrinsically disordered epitope undergoes a disorder–order transition, while in the case of Pal, extensive conformational changes occur in TolB, with localized changes in Pal.\textsuperscript{27,28} We conclude that since single phases predominate in the associations of both ColE9 TBEp and Pal with TolB using the engineered FRET pairs of the present study, the accompanying conformational changes must occur significantly faster than can be detected by stopped flow and thus are not rate-limiting for binding.

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**Scheme 1.** Single-step binding for the TolB–TBEp complex.

**Scheme 2.** Single-step binding for the TolB–Pal complex.
Competitive recruitment of TolB by ColE9 TBEp is efficient and requires Ca\textsuperscript{2+}

Our experiments demonstrate that the kinetic basis for the competitive recruitment of TolB from the TolB–Pal complex by the ColE9 TBEp is the faster association rate of the intrinsically unstructured protein relative to the globular protein. The TolB–TBEp complex associates 7-fold faster (in the presence of Ca\textsuperscript{2+}; Table 2) compared to the formation of the TolB–Pal complex. Combined with the effect of Ca\textsuperscript{2+} on $k_{\text{off}}$ for the TolB–TBEp complex, it is clear that competition for TolB between the intrinsically disordered ColE9 TBEp and Pal can only occur in the presence of Ca\textsuperscript{2+}. This requirement for Ca\textsuperscript{2+} could be demonstrated directly in stopped-flow experiments; dissociation of a 3 μM TolB–Pal complex by a 10-fold excess of ColE9 TBEp could only be accomplished in the presence of Ca\textsuperscript{2+} (Fig. 5).

Antibacterial colicins such as ColE9 assemble a translocon complex at the OM in which a receptor, a translocator, and one or more periplasmic binding partners are brought together to trigger translocation across the OM (Fig. 1).\textsuperscript{31} In the case of ColE9, a key question that we have sought to address in the current work is how the translocation domain is able to recruit TolB given the availability of Pal in the periplasm. This is especially problematic given that a single colicin molecule is sufficient to kill a cell (colicins show pseudo-first-order cell killing kinetics in vivo), and this must be able to compete effectively with Pal. Pal is an abundant protein in the E. coli OM that, in addition to binding TolB in the periplasm, associates with the peptidoglycan layer, with the two binding interactions being mutually exclusive of each other.\textsuperscript{32} The experiment described in Fig. 5 shows that, when in excess, the ColE9 TBEp can dissociate the TolB–Pal complex in the presence of Ca\textsuperscript{2+}. In order to mirror the situation in vivo, where a single copy of the ColE9 TBEp penetrates the periplasm and is faced with the problem of binding TolB in the presence of Pal, we investigated the ability of the ColE9 TBEp to bind TolB in the presence of Pal when all three proteins are held at the same (5 μM) concentration (Fig. 6). In the experiment presented in Fig. 6a, a mixture of ColE9 TBEp (TAMRA labeled) and unlabeled Pal was injected against FAM–TolB in the stopped-flow apparatus, and the changes in donor (495 nm) and acceptor (570 nm) fluorescence emissions were monitored simultaneously using appropriate cutoff filters. Two distinct phases were apparent. The first phase denotes the rapid binding of the ColE9 TBEp to TolB ($k_{\text{obs}}$ at 3.5 s$^{-1}$), where binding is complete within 2 s. Thereafter, a second slower phase appears; here, the TolB–ColE9 TBEp complex dissociates and the TolB–Pal complex forms ($k_{\text{obs}}$ at 0.04 s$^{-1}$). In order to verify that this second rate-limiting step represents Pal binding to TolB from which the ColE9 TBEp has dissociated, we performed an experiment in which a mixture of FAM-labeled Pal and ColE9 TBEp2 (unlabeled) was injected against TMR-labeled TolB (Fig. 6b). Now only a single slow phase was observed for the donor fluorescence that had a rate identical with that of the second phase in Fig. 6a ($k_{\text{obs}}$ at 0.04 s$^{-1}$). We conclude that the intrinsically disordered ColE9 TBE is highly effective at competing with Pal for binding TolB even under equimolar conditions.

![Fig. 5. ColE9 only competes with Pal for TolB binding in the presence of Ca\textsuperscript{2+}. The dissociation of 3 μM TolB–Pal complex through competition with 36 μM ColE9 in the presence (red) or in the absence (blue) of Ca\textsuperscript{2+}. Dissociation is only observed when calcium ions are included in the reaction. The dissociation trace (red) was fitted to a single-exponential equation (black), with residuals shown in the lower panel. The experiments were performed in 50 mM Hepes–NaOH and 50 mM NaCl (pH 7.5) at 37 °C in the presence of 1 mM Ca\textsuperscript{2+} or 10 mM EDTA.](image-url)
work would appear to provide an example of such a competitive advantage, although one that requires additional comment. While the ColE9 TBE has been shown by NMR spectroscopy to be unstructured and flexible, it also contains locally organized hydrophobic clusters that are centered around its two tryptophans, which ultimately bolt the TBE to the TolB surface. Hence, the binding kinetics of the TolB–ColE9 TBE complex must be viewed not only in terms of the binding-induced folding of the IDP but also in terms of the disruption of these hydrophobic clusters preceding epitope association. Nevertheless, the ColE9 TBE is still able to associate faster with TolB than Pal, with the extensive conformational changes in the TolB–Pal complex likely contributing to a slower association rate.

Materials and Methods

Chemicals

TMR was purchased from Invitrogen (Paisley, UK). Hepes buffer (free acid), IPTG, ampicillin, and DTT were purchased from Melford (Ipswich, UK). CaCl₂ and FAM were purchased from Sigma (Poole, UK).

Plasmid mutagenesis

Site-directed mutagenesis was used to introduce cysteine residues in Pal and TolB. PCRs were performed with primer sets purchased by MWG Biotech, and the introduction of the mutations was confirmed by DNA sequencing (Beckman Coulter Genomics). The plasmid pRJ379 (based on pET21d from Novagen) encoding WT TolB (residues 24–439) was the template for Q280C-TolB (pBJW2), while pSL13 (based on pET21a from Novagen) encoding the periplasmic domain of WT Pal (residues 65–173) was the template for S139C-Pal (pKL2). In both cases, the protein sequences were followed by a C-terminal hexahistidine tag.

Protein expression and purification

All proteins were expressed and purified from E. coli BL21(DE3) cells, as described previously by Loftus et al. After Ni-NTA affinity chromatography and size-exclusion chromatography, the purified proteins were dialyzed against 50 mM Hepes–NaOH and 50 mM NaCl (pH 7.0) (buffer A), quantified using the extinction coefficients at 280 nm (ε_TolB = 57,870 M⁻¹ cm⁻¹; ε_Pal = 11,920 M⁻¹ cm⁻¹), and stored at −20 °C until further use.

Fluorophore labeling of Cys mutants

FAM and TMR stocks were prepared in dimethyl sulfoxide and maintained at −20 °C. Each purified single Cys protein was incubated with 10 mM DTT in buffer A (30 min, room temperature). The protein was subsequently desalted in buffer A through either 5-ml HiTrap columns or a 25-ml column of Sephadex G25 resin (GE Healthcare). A 5-fold molar excess of dye was immediately added to the protein solution, and the mix was incubated at 37 °C for 20 min. The labeling reaction was quenched by incubation with 10 mM DTT (30 min, room temperature). The labeled protein was subsequently

![Fig. 6. ColE9 is more efficient than Pal in associating with TolB under stoichiometric conditions. (a) Stopped-flow traces of a 5 μM mixture of WT Pal and TBEp (TAMRA labeled) injected against 5 μM FAM–TolB observed through a 495-nm cutoff filter (top trace; changes in FAM–TolB emission) and a 570-nm cutoff filter (bottom trace; changes in the TAMRA emission of TBEp). (b) Stopped-flow trace of a mixture of 5 μM FAM–Pal and TBEp2 (shorter version of TBEp; see Materials and Methods) injected against 5 μM TMR–TolB observed through a 495-nm cutoff filter. Traces in (a) were fitted to a double-exponential equation (Eq. (2)), yielding the observed rates 3.8 s⁻¹ and 0.04 s⁻¹, while the data in trace (b) were fitted to a single-exponential equation (Eq. (1)) after 2 s (reporting Pal binding), yielding a rate constant of 0.04 s⁻¹. The corresponding residuals to traces (a) (<2.7% systematic error) are shown from top to bottom and are offset for clarity. All traces were obtained in 50 mM Hepes–NaOH and 50 mM NaCl (pH 7.5) at 37 °C in the presence of 1 mM Ca²⁺.

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separated from free dye through desalting in buffer A,
dialyzed overnight against the same buffer, and stored
at −20 °C until further use. The purity and mass of
labeled proteins were verified using electrospray ioniza-
tion mass spectrometry. The I54K–32–54–ColE9 peptide
(32GASDGSGWSENNPWGGGSGSK34, TBEp), which
corresponds to the ColE9 TBE (residues 32–47) with a
five-residue extension (G48–K54), was purchased from
Peptide Protein Research Ltd. The terminal Ile54 in the
ColE9 sequence was mutated to Lys in TBEp in order to
label it with TAMRA at the ε-NH2 group of its side
chain. A shorter peptide, corresponding to only the TBE
sequence of ColE9 32–47 (32GASDGSGWSENNPWG47,
TBEp2), was also used in this study and purchased from
Pepscuticals Ltd. Both peptides were HPLC purified,
and MS analysis showed >98% purity.

Quantification of fluorescently labeled proteins and
TAMRA-labeled peptide

The labeled proteins were quantified by applying the
\( \epsilon_{280} \) values (see the text above) corrected for the
contribution of the fluorophores to the absorbance at
280 nm (\( A_{280} \)). In order to calculate this contribu-
tion, we applied a correction factor for each fluorophore using the
extinction coefficient of the fluorophore at the wavelength
of its maximum absorbance (\( \epsilon_{\text{max}} \)). The applied correction
factors were \( \frac{\epsilon_{280}}{\epsilon_{492}} = 0.19 \) in the case of FAM and \( \frac{\epsilon_{280}}{\epsilon_{505}} = 0.34 \) in the case of TMR. The labeling efficiency
typically ranged between 85% and 97%.

Fluorescence spectroscopy

In order to assess the observed FRET signal, we
recorded the fluorescence emission spectra on a Spex-
FluoroMax 3 spectrophotometer in 50 mM Hepes–NaOH,
50 mM NaCl, and 1 mM CaCl2. Excess TMR–TolB or
TAMRA–TBEp was added to 37 nM FAM–Pal and 780 nM
FAM–TolB, respectively. The fluorescence was excited at
480 nm, and the spectra were recorded above 495 nm, with
the bandwidth set to 3 nm.

Isothermal titration calorimetry

With the use of Microcal ITC200 at 37 °C, titrations
were performed between 18 μM and 30 μM TolB
and between 200 μM and 300 μM Pal, TBEp, or N-terminal
translocation domain of ColE9 (ColE9 T-domain; in
previous calorimetric titrations, the ColE9 T-domain was
shown to bind with identical affinity and thermodynamic
parameters as the unlabeled ColE9 TBEp2 at 20 °C).23 All
proteins were dialyzed against 50 mM Hepes–NaOH,
50 mM NaCl (pH 7.5), and either 1 mM CaCl2 or 10 mM
EDTA. Control experiments in which the titrant protein
was titrated against the buffer were performed and used to
correct for heats of dilution. Data were fitted to a
model of a single set of sites using Microcal Origin.

Stopped-flow fluorescence

Stopped-flow fluorescence experiments were per-
formed on an Applied Photophysics SX18MV setup for
1:1 single mixing and thermostated using a circulating
water bath. An excitation wavelength of 480 nm was used
for the excitation of fluorescein, while the fluorescence
emission was monitored above 495 nm in the case of the
TolB–Pal complex and above 570 nm for the TolB–TBEp
complex using the corresponding cutoff filters. The
manual entrance and exit slits were set to 0.3 mm.
Experiments were carried out at 37 °C in 50 mM Hepes–
NaOH, 50 mM NaCl (pH 7.5), and either 1 mM CaCl2 or
10 mM EDTA. A total of 4000–12,000 data points were
collected over the course of each reaction, and all data
collected before 2 ms were not included in the analysis.
With the exception of the experiments described in Fig. 6,
all association data were collected under pseudo-first-
order conditions. Pal was held constant at 1 μM, while
TolB varied between 5 μM and 40 μM; in the case of TBEp,
each binding partner was held constant at 2 μM, while
the other varied between 10 μM and 40 μM. The pseudo-first-
order kinetic traces were fitted to a single-exponential
rate equation (Eq. (1)), where \( F \) is the fluorescence at
time \( t \), \( \Delta F \) is the total fluorescence change, \( k_{\text{obs}} \) is
the observed rate constant, and \( F_e \) is the end-point
fluorescence) by non-linear least-squares regression using the
Workstation software (Applied Photophysics Ltd.). Pseudo-first-order
plots of \( k_{\text{obs}} \) against excess [protein] were used to
determine the bimolecular association rate constant (\( k_{\text{on}} \)) and
the dissociation rate constant (\( k_{\text{off}} \)) (Schemes 1 and 2)
by performing linear least-squares analysis using Kaleida-
graph (Synergy software). Dissociation experiments (fitted to a
single-exponential equation) were performed through
competition between 6 μM TolB–TBEp complex and
180 μM or 260 μM unlabeled WT ColE9 T-domain, and
between 3 μM TolB–Pal complex and 36 μM or 45 μM
unlabeled WT Pal. Competition experiments were also
performed between 3 μM TolB–Pal complex and 36 μM
WT ColE9 and fitted to a single-exponential equation. In
the stopped-flow experiments of the Pal and ColE9 TBE
mixture (WT Pal/TBEp versus FAM–TolB, or FAM–Pal/
TBEp2 versus TMR–TolB), all proteins were set to 5 μM,
and the conditions were as described above in the
presence of 1 mM CaCl2. In order to acquire the
corresponding rate constants, we fitted the single-phase
trace of FAM–Pal versus TMR–TolB to Eq. (1), while
the two-phase traces of TBEp versus FAM–TolB were fitted to
a double-exponential equation (Eq. (2)):

\[
F = \Delta F_{\text{exp}}(-k_{\text{obs}}t) + F_e
\]

\[
F = \Delta F_1\exp(-k_{1}t) + \Delta F_2\exp(-k_{2}t) + F_e
\]

Acknowledgements

We thank Berni Strongarith (deceased) and Dr.
Andrew Leech for their help with biophysical
experiments performed at the Molecular Interac-
tions Laboratory of the Technology Facility in York.
This work was funded by the Biotechnology and
Biological Sciences Research Council of the UK
(grant BB/G020671/1) and The Wellcome Trust.
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