Cell entry mechanism of enzymatic bacterial colicins: Porin recruitment and the thermodynamics of receptor binding

Nicholas G. Housden*, Steven R. Loftus*, Geoffrey R. Moore†, Richard James‡, and Colin Kleanthous*§

*Department of Biology (Area 10), P.O. Box 373, University of York, York YO10 5YW, United Kingdom; †School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich NR4 7TJ, United Kingdom; and ‡Molecular Medical Sciences, Institute of Infection, Immunity, and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom

Edited by Thomas J. Silhavy, Princeton University, Princeton, NJ, and approved August 10, 2005 (received for review May 2, 2005)

Binding of enzymatic E colicins to the vitamin B_{12} receptor, BtuB, is the first stage in a cascade of events that culminate in the translocation of the cytotoxic nuclease into the *Escherichia coli* cytoplasm and release of its tightly bound immunity protein. A dogma of colicin biology is that the toxin coiled-coil connecting its functional domains must unfold or unfurl to span the periplasm, with recent reports claiming this reaction is initiated by receptor binding. We report isothermal titration calorimetry data of BtuB binding the endonuclease toxin ColE9 and a disulfide form (ColE9$^{S\text{-}S}$) where unfolding of the coiled-coil is prevented and, as a consequence, the toxin is biologically inactive. Contrary to expectation, the thermodynamics of receptor binding, characterized by large negative values for TΔS, are identical for the two colicins, arguing against any form of BtuB-induced unfolding. We go on to delineate key features of the ''colicin translocon'' that assembles at the cell surface after BtuB binding by using a complex of histidine-tagged Im9 bound to ColE9$^{S\text{-}S}$. First, we show that the porin OmpF is recruited directly to the BtuB·colicin complex to form the translocon. Second, recruitment is through the natively unfolded region of the colicin translocation domain, with this domain likely having two contact points for OmpF. Finally, the immunity protein is not released during its assembly. Our study demonstrates that although colicin unfolding is undoubtedly a prerequisite for *E. coli* cell death, it must occur after assembly of the translocon.

colicin | toxin | outer membrane | translocation | native disorder

Protein translocation involves the movement of proteins between cellular compartments. Essential for secretion, protein translocation also underpins the ability of microbial toxins to penetrate cells. Both secretion and toxin import rely on protein assemblies to provide the conduit for the translocating polypeptide. In contrast to secretion, however, where these conducting complexes have evolved to facilitate translocation, protein toxins parasitize normal host cell processes, exemplified by the translocation of microbial toxins into eukaryotic cells (1) and by the import of bacteriocins into prokaryotes (2). Primarily expressed during times of stress, bacteriocins are protein antibiotics made by most species of bacteria that play a pivotal role in microbial colonization, competition, and diversification (3, 4). Given the potency of bacteriocins (they exhibit single-hit kinetics) and their exquisite cellular specificity, understanding how they penetrate bacterial cells offers new opportunities for the design of antimicrobial agents and provides novel ways of probing the function of host proteins that are parasitized by them. However, bacteriocin translocation is poorly understood. In the present work, we answer some of the key questions concerning early events in the cellular import of colicins, bacteriocins specific for *Escherichia coli*.

Colicins enter cells by coopting a number of proteins in the *E. coli* outer envelope, including an outer membrane receptor and one or more periplasmic proteins (5, 6). The outer membrane receptors are nutrient receptors, normally engaged either in the passive import of small molecules or in the active transport of complex molecules such as chelated iron or vitamin B_{12} (cobalamin) into the cell. Consequently, such receptor systems are an ''Achilles heel'' for the bacterium, exploited by filamentous bacteriophage and colicins as a gateway into the cell (7).

As well as traversing the outer membrane, colicins must also find a way through the periplasm. Accomplishing both feats involves recruitment of either the TonB/ExbB/ExbD complex, specific for group B colicins such as Ia, Ib, B, and D, or the TolQRAB/Pal complex, utilized by group A colicins such as E2-E9, N, and A (5, 8). Both the Ton and Tol systems are coupled to the protonmotive force across the inner membrane, acting as energy transducers for active transport (Ton) or maintenance of outer envelope integrity (Tol) (9). It is generally thought that colicins tap into the energy associated with these systems to translocate into cells although how this mechanism is accomplished is not known.

Colicins most commonly elicit cell death through depolarization of the inner membrane by an ionophore (Ia, Ib, E1, N, A, and B) or through nuclease action that targets cytoplasmic DNA (E2 and E7-E9), rRNA (E3, E4, and E6) or tRNA (E5 and D). We have been studying the cell-entry mechanism of that, as with all E colicins, uses the cobalamin receptor BtuB as its primary outer membrane receptor (10). ColE9 is a 60-kDa group A colicin that delivers a metal-dependent endonuclease (related to the apoptotic nuclease, CAD) to the cytosol, causing cell death through nonspecific degradation of the bacterial genome (6). Colicins have hairpin-like structures, characterized by that of the rRNase colicin ColE3 (11) (Fig. 1) and the ionophore ColIa (14). These toxins comprise a central receptor-binding (R) domain flanked by N-terminal translocation (T) and C-terminal cell-killing domains, the latter two in close proximity and separated from the R domain by a long coiled-coil. Colicins are thought to unfold or unfurl to allow them to span the periplasm while simultaneously contacting the outer membrane. Such large-scale structural changes are supported by the data of Penfold *et al.* (15), who showed that a constraining disulfide bond engineered across the ColE9 R domain (Fig. 1) abolished toxin activity that could be restored on reduction with DTT. Although colicin unfolding is generally accepted as a part of the uptake mechanism, uncertainty remains as to when such structural changes occur, with one recent suggestion from Cramer and coworkers that colicin unfolding is induced by BtuB binding (12, 16).

Focusing on the DNase ColE9, we have used isothermal titration calorimetry (ITC) to test the hypothesis that E colicin binding to the BtuB receptor causes toxin unfolding. We find

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ColE9, colicin E9; Im, immunity protein; β-OG, *n*-octyl-β-D-glucopyranoside; ITC, isothermal titration calorimetry; ColE9$^{S\text{-}S}$, ColE9 with a disulfide bond between residues 324 and 447 within the R domain.

§To whom correspondence should be addressed. E-mail: ck11@york.ac.uk.

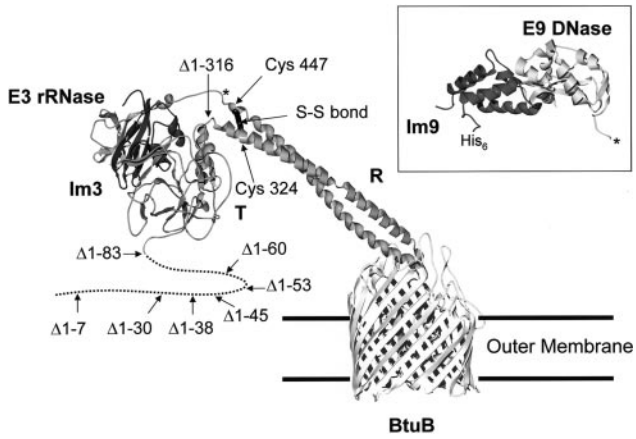

Fig. 1. Structure of ColE3/Im3 modeled on that of the BtuB·ColE3 R-domain complex (11, 12). The figure highlights the positions of the R and T domains of the enzymatic E colicin, the C-terminal E3 rRNase bound to Im3, and the locations of the T domain deletions that were engineered into an R-domain disulfide bond variant of ColE9 (ColE9$^{S\text{-}S}$). (*Inset*) Structure of the ColE9 DNase/Im9 complex (13). The asterisk shows where on the full-length colicin the domain would be attached in ColE9/Im9. ColE3 and ColE9 are 92% identical in sequence up to this point.

that the thermodynamics of colicin BtuB complex formation are not consistent with toxin unfolding but rather with increased rigidification of the receptor. It is also unlikely colicins unfold during assembly of the outer membrane translocon, which we show involves the recruitment of OmpF to the BtuB·colicin complex through a natively disordered domain of the toxin. We conclude that the mechanism of colicin import into bacterial cells needs to be reappraised in light of these data.

Materials and Methods

Protein Purification. BtuB was purified by using a protocol modified from that used by Law *et al.* (17). The cell pellet from a 5-liter culture of *E. coli* TNE012 cells (K12 *tsx*$^-$, *ompA*$^-$, *ompB*$^-$) transformed with pNP278 (containing *btuB* under the control of the arabinose promoter) was resuspended in 35 ml of 10 mM Tris·HCl, pH 8.0/0.25% (wt/vol) lithium diiodosalicylate in the presence of Complete protease inhibitor mixture (Roche Diagnostics) and sonicated on ice. Cell debris was removed by centrifugation at 5,000 × *g* for 10 min, followed by centrifugation of the supernatant at 200,000 × *g* for 1 h at 4°C. Inner membrane proteins were extracted from the total membrane pellet through homogenization in 56 ml of 10 mM Tris·HCl, pH 8.0/0.25% (wt/vol) lithium diiodosalicylate/2% (vol/vol) Triton X-100. After centrifugation at 200,000 × *g* for 1 h, the outer membrane pellet was resuspended in 56 ml of 10 mM Tris·HCl, pH 8.0, followed by centrifugation at 200,000 × *g* for 1 h at 4°C. Outer membrane proteins were solubilized in 56 ml of 10 mM Tris·HCl, pH 8.0, containing 1% (wt/vol) *n*-octyl-β-D-glucopyranoside (β-OG), centrifuged at 200,000 × *g* for 1 h at 22°C, and the extract was loaded onto a 4-ml DEAE-Sepharose anion exchange column equilibrated in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.54% β-OG, 100 mM LiCl. After a 40 ml wash, BtuB was eluted from the DEAE-Sepharose column with a 0.1–1.0 M LiCl gradient. The final gel-filtration step of Law *et al.* (17) was found to diminish the quantity of LPS associated with BtuB, with a concurrent 50- to 100-fold decrease in the ability of exogenous BtuB to protect susceptible cells from Col cytotoxicity. Although efficient restoration of BtuB-mediated protection could be achieved by the addition of LPS, the gel filtration step was omitted because of the high purity of BtuB after anion exchange. Purified pooled fractions were stored at −20°C.

Wild-type ColE9/immunity protein 9 (Im9) and full-length and truncated ColE9 with a disulfide bond between residues 324 and 447 within the R domain (ColE9$^{S\text{-}S}$)/Im9 complexes (constructed by PCR mutagenesis) were purified from pET21a BL21(DE3) cell lysate through the hexa-histidine tag at the C terminus of the immunity protein by using nickel affinity chromatography, followed by size exclusion chromatography, as described in ref. 18.

All protein concentrations were determined although A_{280} measurements by using theoretical molar extinction coefficients calculated from amino acid sequences.

Isothermal Titration Calorimetry. ITC measurements were performed by using a MicroCal VP-ITC thermostated at 25°C. Proteins were dialyzed overnight into 25 mM Tris·HCl, pH 7.5/150 mM NaCl/1% β-OG (supplemented with either 5 mM EGTA or 5 mM $CaCl_2$) by using a 25-kDa molecular mass cutoff Spectra/Por membrane. BtuB was typically present in the sample cell at a concentration of 1.2 μM with 16 μM ColE9/Im9 in the syringe. After an initial 2-μl injection, 29 aliquots of 10 μl were titrated from the syringe into the sample cell. Binding isotherms were analyzed by using the manufacturer's software.

Purification of ColE9/Im9 Outer Membrane Protein Complexes. Cell pellets from a 2.5-liter arabinose-induced pNP278 B^{E}3000 (*ompF*$^+$, *ompC*$^-$) culture were resuspended in 10 mM Tris·HCl, pH 8.0/0.25% (wt/vol) lithium diiodosalicylate in the presence of 15 mg of bait protein (ColE9$^{S\text{-}S}$/Im9 or derivative) (15) and Complete protease inhibitor mixture to give a final volume of ≈35 ml. After incubation at 4°C for 1 h, cells were lysed by using a French press and insoluble cell debris was removed by centrifugation at 5,000 × *g* for 10 min. The total membrane pellet was harvested by centrifugation at 200,000 × *g* for 1 h at 4°C. Inner membrane proteins were extracted as described above. Outer membrane proteins in complex with ColE9$^{S\text{-}S}$/Im9 were resuspended from the remaining pellet in 20 mM Tris·HCl, pH 8.0/25 mM NaCl/5 mM imidazole/5 mM EDTA/1% β-OG, incubating for 12 h at 37°C with shaking before centrifugation at 200,000 × *g* for 1 h at 22°C. The extract was buffer exchanged to remove EDTA and loaded onto a 1-ml nickel-charged HisTrap HP column (Amersham Pharmacia Biosciences) to capture the ColE9$^{S\text{-}S}$/Im9 complex and associated proteins, utilizing the hexa-histidine tag on the C terminus of Im9. Unbound proteins were washed from the column with 10 column volumes of 20 mM Tris·HCl, pH 8.0/25 mM NaCl/5 mM imidazole/1% β-OG, followed by elution of bound proteins with a 5–300 mM imidazole gradient. Eluted fractions were analyzed by SDS/PAGE, with bands of interest being excised from the gel, trypsin digested, and identified through MALDI-TOF MS (Applied Biosystems 4700).

***In Vivo* Cytotoxicity Assay.** Fifty-milliliter shake flask cultures of LB supplemented with 100 μg/ml ampicillin and 5 μg/ml ColE9$^{S\text{-}S}$/Im9 were inoculated with 400 μl of colicin susceptible JM83 pTrc99A overnight culture. Cultures were incubated at 37°C, with bacterial growth being monitored through OD_{600} measurements at 30-min intervals. Two hours after inoculation, ColE9$^{S\text{-}S}$/Im9 constructs were activated through the addition of DTT to a final concentration of 5 mM. Bacterial growth was monitored for an additional 5.5 h.

Results

Thermodynamics of BtuB·ColE9/Im9 Complex Formation and the Influence of Ca^{2+}. Ca^{2+} ions are known to bind to extracellular loops of the BtuB receptor creating the high-affinity binding site for cobalamin (19). The published structure for the E colicin R-domain bound to BtuB is in the absence of Ca^{2+} ions (12). We performed ITC experiments in the presence and absence of Ca^{2+}

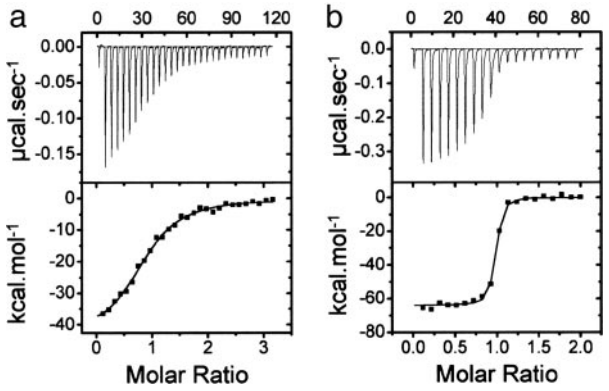

Fig. 2. Representative ITC binding curves for the formation of the BtuB·ColE9/Im9 complex. (*a*) Titration of a 16 μM ColE9/Im9 complex into 1.2 μM BtuB in 25 mM Tris·HCl, pH 7.5/150 mM NaCl/1% β-OG in the presence of 5 mM EGTA. (*b*) Equivalent titration of 16 μM ColE9/Im9 complex into 1.2 μM BtuB in 25 mM Tris·HCl, pH 7.5/150 mM NaCl/1% β-OG in the presence of 5 mM $CaCl_2$. Data were fitted to a single binding-site model. Derived thermodynamic parameters for these complexes are listed in Table 1.

ions to probe the thermodynamics of colicin·BtuB interactions and to establish whether they were compatible with receptor-induced unfolding of the toxin. In addressing these issues, we initially used intact ColE9 bound to its immunity protein, Im9, because it is in the form of a heterodimeric complex that enzymatic E colicin bind to BtuB (ref. 20 and Fig. 1).

In the presence of 5 mM EGTA, the affinity of the BtuB·ColE9/Im9 complex was relatively modest, with a K_d of 153 nM, but increased 65-fold to 2.4 nM in the presence of 5 mM Ca^{2+} ions (Fig. 2). A similar relative increase in affinity was noted by Mohanty *et al.* (21) in their spectroscopic study of a 34 amino acid R-domain peptide binding to BtuB, although the measured affinities were >1 order of magnitude weaker. Calorimetric data for ColE9/Im9 binding to BtuB are summarized in Table 1. The data show that the free energy of binding in the absence of Ca^{2+} ($\Delta G = -9.3$ kcal·mol^{-1}) is the net result of an extremely large and favorable enthalpy ($\Delta H = -42$ kcal·mol^{-1}) but an unfavorable entropy ($T\Delta S = -33$ kcal·mol^{-1}). We also found that the presence of the nuclease-bound immunity protein had little effect on these parameters (Table 1), suggesting receptor binding does not disrupt interactions to the inhibitor. This interpretation was confirmed by analytical gel-filtration chromatography of the BtuB·ColE9/Im9 complex, which showed that Im9 remained bound to the colicin (data not shown). The thermodynamic parameters are consistent with significant ordering taking place within the confines of the complex and the formation of several intermolecular hydrogen bonds. Both suppositions are borne out by the structure of the E colicin R-domain·BtuB complex; several extracellular loops of BtuB become ordered on binding to the E colicin R domain, resulting in the burial of 1,500 Å^2 accessible surface area and the formation of nine intermolecular hydrogen bonds.

The thermodynamic basis for the increase in affinity of the BtuB·ColE9/Im9 complex in the presence of Ca^{2+} ($\Delta G = -11.8$ kcal·mol^{-1}) is due to the reaction becoming even more exothermic ($\Delta H = -63$ kcal·mol^{-1}), with a concomitant increase in negative entropy ($T\Delta S = -51$ kcal·mol^{-1}). These data imply that colicin binding to Ca^{2+}-bound BtuB causes even greater structural remodeling of the binding site. Although no structure for an R-domain·BtuB·Ca^{2+} complex is yet available, we note that the conformations of two loops in the Ca^{2+}-bound state of BtuB differ significantly from those in the colicin R-domain-bound state (12, 22). This observation suggests that loop conformations in the R-domain·BtuB·Ca^{2+} complex will differ to those seen in the absence of Ca^{2+}.

Thermodynamics of BtuB·ColE9$^{S\text{-}S}$/Im9 Complex Formation Discount Receptor-Induced Unfolding of the Colicin. The significant negative entropy associated with ColE9/Im9 binding the BtuB receptor is counterintuitive. Kurisu *et al.* (12) have argued that enzymatic E colicin binding to BtuB results in disordering of the N- and C-terminal ends of the R-domain, which might be expected to yield a positive entropy in ITC experiments. The absence of such an effect could, however, be explained by the negative entropy associated with ordering of BtuB extracellular loops masking the positive entropy associated with E colicin unfolding. To assess the contribution of unfolding of the colicin R-domain to the thermodynamics of receptor binding, we used the conformationally restricted ColE9$^{S\text{-}S$/Im9 complex in ITC experiments. ColE9$^{S\text{-}S$/Im9 has an inactivating disulfide bond engineered across the distal end of the R-domain, thereby preventing unfolding of its coiled-coil structure (Fig. 1 and ref. 15). As with wild-type colicin, binding of ColE9$^{S\text{-}S$/Im9 to BtuB is calcium dependent. Surprisingly, however, the values for enthalpy, entropy, and free energy ($\pm Ca^{2+}$) are almost identical to those of the wild-type toxin (Table 1). Hence, the calorimetric data for ColE9 and ColE9$^{S\text{-}S$ binding to BtuB demonstrate that no global changes occur in the conformation of the toxin, ruling out the possibility that binding to BtuB induces colicin unfolding.

Recruitment of OmpF to the BtuB·ColE9/Im9 Complex by the Natively Disordered Colicin T Domain. After binding to BtuB, specific N-terminal sequences within E colicins must contact the Tol/Pal complex in the periplasm in order for the toxin to translocate into the bacterium. In the case of the enzymatic colicins E3 and E9, residues D^{35}GSGW^{39} comprise the so-called ''TolB box'' because they bind TolB and are essential for biological activity (23, 24). Importantly, the TolB box is part of a natively disordered domain of the T domain at the extreme N terminus of the toxin (25), with similarly unstructured regions found in other colicins (26). A central question then is how these sequences are able to traverse the outer membrane in order that they bind TolB. Enzymatic E colicins are known

Table 1. Thermodynamic parameters for ColE9 binding the BtuB receptor

	Plus 5 mM EGTA			Plus 5 mM $CaCl_2$		
Colicins	ColE9/Im9	ColE9	ColE9$^{S\text{-}S$/Im9	ColE9/Im9	ColE9	ColE9$^{S\text{-}S$/Im9
N	0.89 ± 0.01	0.99 ± 0.01	0.82 ± 0.01	0.94 ± 0.01	0.94 ± 0.04	0.90 ± 0.02
K_d, nM	153 ± 28	50.4 ± 12.5	71.8 ± 3.3	2.1 ± 0.2	2.5 ± 1.7	1.0 ± 0.1
ΔG, kcal·mol^{-1}	−9.3 ± 0.1	−10.1 ± 0.1	−9.7 ± 0.0	−11.8 ± 0.1	−11.2 ± 0.2	−12.3 ± 0.0
ΔH, kcal·mol^{-1}	−42.0 ± 2.4	−39.6 ± 2.6	−40.0 ± 1.0	−62.5 ± 2.3	−58.5 ± 1.9	−67.6 ± 0.1
$T\Delta S$, kcal·mol^{-1}	−32.8 ± 2.5	−29.6 ± 2.8	−30.2 ± 1.1	−50.6 ± 2.1	−46.7 ± 2.3	−55.0 ± 0.2

ITC parameters for ColE9, ColE9/Im9 and ColE9$^{S\text{-}S$/Im9 binding the BtuB receptor in the presence and absence of calcium at 25°C in 25 mM Tris·HCl, pH 7.5/150 mM NaCl/1% (wt/vol) β-OG. The errors shown are those from duplicate measurements.

to require porins such as OmpF, in addition to BtuB, to elicit cell death (27), suggesting that OmpF may offer a route into the periplasm. Consistent with such an entry mechanism, the channel activity of OmpF molecules reconstituted into planar lipid bilayers is blocked by N-terminal sequences from ColE3, implying that the lumen of the porin becomes occluded (12). Currently, however, there is no evidence that an enzymatic E colicin bound to BtuB in the outer membrane is able to recruit OmpF, which would be a requirement for such a mechanism to operate.

We addressed the issue of porin recruitment through affinity purification of colicin-bound protein complexes extracted from the outer membrane of *E. coli*, capitalizing on the inability of ColE9$^{S\text{-}S}$ to enter and kill bacterial cells while still binding BtuB. Purification of membrane-bound receptor complexes was expedited by the hexa-histidine tag that had been engineered onto the C terminus of Im9 in the ColE9$^{S\text{-}S}$/Im9 complex utilized throughout this study (Fig. 1). Milligram quantities of the toxin were added to *E. coli* B^{E}3000 cells, and detergent solubilized toxin-bound complexes purified by Ni^{2+} affinity chromatography, as described in *Materials and Methods*. A single absorbance peak (at 280 nm) was observed upon elution of bound protein from the nickel-affinity column with a linear imidazole gradient (Fig. 3*a*). After SDS/PAGE and MALDI-TOF MS of trypsin-digested gel bands, this peak was shown to contain BtuB, Col E9, Im9, and OmpF. The amount of trimeric OmpF recovered bound to the BtuB·ColE9 complex was generally substoichiometric (estimated by densitometry of SDS-polyacrylamide gels as $\approx 0.3 \pm 0.2$ from eight separate extractions), the low yield likely reflecting a combination of weak binding and stringent extraction procedures. Our data provide direct evidence that OmpF binds to the BtuB·ColE9/Im9 complex in the *E. coli* outer membrane.

We next delineated the regions of ColE9$^{S\text{-}S}$ responsible for the recruitment of OmpF by using affinity purification as an assay. Deletion of residues 1–316 (Δ1–316 ColE9$^{S\text{-}S}$/Im9), encompassing both the structured and unstructured regions of the T domain (Fig. 1), still resulted in the purification of BtuB from *E. coli* membranes but OmpF was lost entirely from the complex (Fig. 3*b*). Importantly, deletion of just the disordered T-domain sequences (Δ1–83 ColE9$^{S\text{-}S}$/Im9) also resulted in the complete loss of OmpF from the BtuB·colicin complex (Fig. 3*c*). A critical role therefore of the natively disordered 83 amino acids in enzymatic colicins is to recruit OmpF to the BtuB·ColE9/Im9 complex in the outer membrane.

Diminished OmpF Recruitment to the BtuB·ColE9/Im9 Outer Membrane Complex Decreases the Efficiency of Cell Killing. To determine which regions of the disordered T domain were necessary for OmpF recruitment, seven additional truncations were made, removing residues 1–7 (Δ1–7 ColE9$^{S\text{-}S}$/Im9), 1–30 (Δ1–30 ColE9$^{S\text{-}S}$/Im9), 1–38 (Δ1–38 ColE9$^{S\text{-}S}$/Im9), 1–45 (Δ1–45 ColE9$^{S\text{-}S}$/Im9), 1–53 (Δ1–53 ColE9$^{S\text{-}S}$/Im9), 1–60 (Δ1–60 ColE9$^{S\text{-}S}$/Im9) and 60–80 (Δ60–80 ColE9$^{S\text{-}S}$/Im9). Recruitment of OmpF was lost entirely in both the 1–60 and 60–80 deletions (data not shown). Intriguingly, there was little difference between the 7 and 53 residue deletions where OmpF was recruited but in reduced yield. Densitometric scanning of SDS-polyacrylamide gels from duplicate experiments indicated that, relative to full-length ColE9$^{S\text{-}S}$/Im9 extractions carried out on the same batch of cells, only $14 \pm 4\%$, $15 \pm 2\%$, $9 \pm 5\%$, $10 \pm 5\%$, and $9 \pm 5\%$ OmpF was affinity-purified for Δ1–7 ColE9$^{S\text{-}S}$/Im9, Δ1–30 ColE9$^{S\text{-}S}$/Im9, Δ1–38 ColE9$^{S\text{-}S}$/Im9, Δ1–45 ColE9$^{S\text{-}S}$/Im9, and Δ1–53 ColE9$^{S\text{-}S}$/Im9 respectively (data not shown). The data imply that distinct, noncontiguous regions of the disordered T domain of the Col are required for OmpF recruitment that straddle the TolB box (residues 35–39).

We next determined what effect these deletions had on the cell killing activity of ColE9. Cultures of susceptible *E. coli* JM83 cells were grown in shaking flasks and the bactericidal effects of the different ColE9$^{S\text{-}S}$ constructs monitored under reducing conditions (Fig. 4). As previously published, oxidized ColE9$^{S\text{-}S}$ has no cell killing activity, whereas the reduced form of the toxin has wild-type biological activity (15). Reduced Δ1–60 ColE9$^{S\text{-}S}$/Im9 (Fig. 4) and Δ60–80 ColE9$^{S\text{-}S}$/Im9 were both inactive. The former was expected because it lacks a TolB box; however, the latter suggests that the loss of activity is due to the inability to recruit OmpF. The reduced forms of Δ1–7 ColE9$^{S\text{-}S}$/Im9 and Δ1–30 ColE9$^{S\text{-}S}$/Im9, both of which contain TolB boxes, had identical biological activities wherein cell death took longer to initiate (Fig. 4). These data reveal that deletion of OmpF contacting residues N-terminal to the TolB box decreases porin recruitment and the efficiency of *E. coli* cell killing, whereas removal of OmpF contacting sequences C-terminal to the TolB box abolishes both.

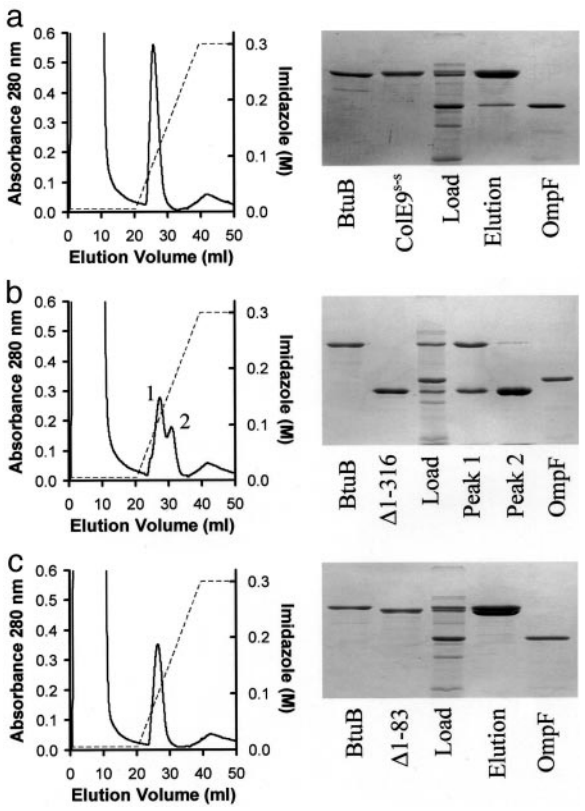

Fig. 3. OmpF is recruited to the BtuB·ColE9$^{S\text{-}S}$/Im9 complex by the disordered colicin T domain. Outer membrane protein complexes were extracted from *E. coli* B^{E}3000 membranes and purified in the presence of full length ColE9$^{S\text{-}S}$/Im9 (*a*), Δ1–316 ColE9$^{S\text{-}S}$/Im9 (*b*) or Δ1–83 ColE9$^{S\text{-}S}$/Im9 (*c*) and loaded onto 1-ml Ni-charged HisTrap columns equilibrated in 20 mM Tris·HCl, pH 8.0/25 mM NaCl/5 mM imidazole/1% (wt/vol) β-OG. The chromatograms show protein complexes eluted from the column with a 5–300 mM imidazole gradient, while monitoring the absorbance of the eluent at 280 nm, and have been corrected for buffer contributions. In *a* and *c*, single peaks were observed, whereas in *b*, two peaks eluted from the column, the latter most likely due to a weakening of the interaction between ColE9 and BtuB in this deletion construct. The 10% SDS-polyacrylamide gels adjacent to each chromatogram include the peak fraction(s) from elution, the outer membrane extract loaded onto each column, and controls of full-length or truncated colicins, BtuB and OmpF. In all cases, BtuB and ColE9 were recovered but OmpF was lost when the colicin lacked the disordered T domain. With the low percentage SDS-polyacrylamide gels used to resolve extracted proteins, Im9 was not separated from the dye front and is not observed. Bands in the eluted fractions were excised, digested with trypsin, and the proteins identified unambiguously by MALDI-TOF mass spectrometry (data not shown).

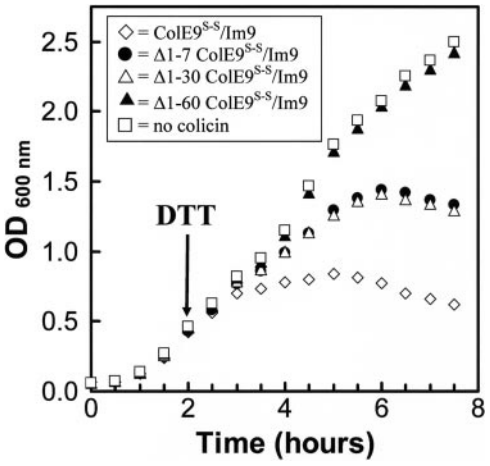

Fig. 4. Colicins deficient in OmpF recruitment are less effective at killing *E. coli* K-12 cells. ColE9^{S-S}/Im9 and the T-domain deletions Δ1–7, Δ1–30, and Δ1–60 ColE9^{S-S}/Im9 were added to separate flasks of *E. coli* JM83 cells at the point of inoculation (0 h). All cultures behaved identically until the addition of DTT (5 mM) when the cultures had reached an optical density of ≈0.5 (as indicated by the arrow), reducing the R-domain disulfide bond and, hence, initiating cell killing. Δ1–60 was inactive in this assay, whereas full-length ColE9^{S-S}/Im9 showed wild-type colicin cytotoxic activity. Δ1–7 and Δ1–30 ColE9^{S-S}/Im9 constructs had identical cell-killing profiles that were significantly reduced relative to full-length Col.

Discussion

E Colicin R Domain Unfolding Likely Occurs After BtuB Receptor Binding. The elongated structure of a colicin is thought to be important in allowing the toxin to traverse the periplasm during translocation. Because each coil in an enzymatic E colicin such as ColE3 or ColE9 is ≈100 Å in length and the periplasm is >150 Å, then to span the periplasm, the coiled-coil regions linking the R, T, and cell-killing domains must unfold or unfurl. Consistent with such unfolding, a disulfide bond engineered across the coiled-coil, but distal to the BtuB binding site, abolishes biological activity of the colicin. A key question therefore is when such unfolding occurs.

In their structure of the ColE3 R-domain bound to BtuB, Kurisu *et al.* (12) observed a systematic increase in main-chain displacement of the coiled-coil relative to the unbound state of ColE3, and that the final 8–9 amino acids of each end of the R domain were disordered. These observations were interpreted as evidence of receptor-induced unfolding of the colicin coiled-coil. We tested whether BtuB binding induces E colicin R-domain unfolding through calorimetric measurements by using the native, intact ColE9/Im9 complex in the presence and absence of Ca^{2+}. The magnitudes of the enthalpic and entropic parameters underpinning BtuB·ColE9/Im9 complex formation are among the largest found in the ITC literature for a protein–protein interaction. Comparable figures have been reported for partially or natively unstructured proteins that fold on binding to their partner, where the observed ΔH and ΔS values are the summation of the energy changes of binding and folding (28, 29). X-ray crystal structures have shown that R-domain·BtuB complex formation is accompanied by increased structure in the BtuB extracellular loop regions, with residues 229–240 and 278–287 being resolved in the R135 bound form (12) but not the apo form (13) of BtuB. The similarity of the thermodynamic parameters for the equivalent BtuB complexes of wild-type and structurally restrained ColE9^{S-S}/Im9 implies that any increase in rigidity or structure is likely to be localized to the receptor itself. We conclude from these data that the E colicin R domain does not undergo receptor-induced unfolding.

How does an enzymatic E colicin unfold if not induced by BtuB? We speculate that unfolding occurs after BtuB binding, which necessitates that the toxin be actively displaced from its primary receptor-binding site before cell entry. Because this process would require an input of energy, such displacement could provide an explanation as to why the energized Tol system is coopted for colicin import. Furthermore, given that the tip of the helical hairpin linking each of the coils forms the majority of the interactions with BtuB, receptor displacement would permit a fully extended R-domain structure to form, thereby allowing the colicin to traverse the periplasm.

Assembly of a Colicin Translocon and the Role of Native Disorder. The structural organization of the BtuB·R-domain complex suggests that BtuB itself is not involved in translocation (12). Colicin binding does not displace the plug domain, which occludes completely the central pore of the 22 β-strand receptor, thereby negating the pore as a possible route into the periplasm. In addition, the toxin is positioned at a 45° angle relative to the membrane surface, placing the cytotoxic and T domains away from BtuB in a trajectory that suggests additional partners are being sought to enable translocation (Fig. 1). We have demonstrated that the porin OmpF is recruited directly to the BtuB·colicin complex through a disordered T domain to form a "colicin translocon," as had been postulated but not shown previously. Binding of OmpF to BtuB-bound colicin appears to be weak, which would explain why it has not been possible to demonstrate the assembly of a similar complex simply by using purified components that, for example, survives gel-filtration chromatography (17). The success of the affinity purification approach used in the present study is likely due to the *in vivo* assembly of the ColE9/Im9·BtuB complex on the cell surface, followed by the extraction of the entire complex from the same membrane.

OmpF is one of the most abundant proteins in the bacterium (>100,000 copies per cell), whereas BtuB is expressed at very low levels (≈200 copies per cell). Hence, enzymatic E colicins use high-affinity binding to a rare outer membrane nutrient receptor as a route for finding an abundant porin as a translocator. Another important conclusion that can be reached about the assembly of the colicin translocon is that it also occurs in the absence of any unfolding of the coiled-coil R domain. Why is OmpF recruited to the BtuB·ColE9/Im9 complex, and how does this association facilitate binding TolB in periplasm? Each monomer of an OmpF trimer is a 16-stranded β-barrel with a central, water-filled transmembrane channel that allows the passive diffusion of relatively small (<700 Da) hydrophilic molecules across the outer membrane. At its narrowest point, the OmpF pore is 7 × 11 Å (30), which is wide enough to allow the passage of an unfolded polypeptide, such as the disordered T domain of an enzymatic E colicin, into the periplasm. The rationale for using OmpF in this way, apart from a lumen that traverses the outer membrane, is that trimeric porins such as OmpF are known to associate with TolB (31). Recruitment of OmpF by a BtuB-bound colicin therefore ensures the toxin can engage the energized Tol system.

Natively unfolded proteins are gaining an increasing prominence in biology, shown to be important in cellular processes such as signaling, transcription, and translation (32, 33). Characterized by low sequence complexity (41% Gly and 17% Ser), heteronuclear NMR spectroscopy has shown that the first 83 amino acids of the enzymatic E colicin T domain has the conformational properties classically ascribed to natively disordered polypeptides (25).

Native disorder provides the capacity for multiple binding interactions because several linear epitopes can be accommodated along the polypeptide chain. In the case of the ColE9 T domain, the TolB box is located between residues 35–39 (34),

with NMR showing the binding site extends to at least residue 43 (25). The present work suggests that, in addition, the disordered T domain of an enzymatic colicin contains two regions that comprise the OmpF binding site. One region is contained within the first 7 amino acids of the disordered T domain, supported by the work of Zakharov *et al.* (35), who have shown these residues are important for blocking OmpF channels in planar lipid bilayer experiments. The present work indicates the loss of this site decreases OmpF recruitment to the colicin translocon and reduces the efficiency of cell killing but does not abolish it (Fig. 4). The second OmpF-binding site lies between residues 54–80 as mapped through the ability of deletion constructs made in this study (Δ1–38, Δ1–45, and Δ1–53 ColE9$^{S\text{-}S}$/Im9) to recruit OmpF, whereas deletion of residues 1–60 or 60–80 of the colicin result in the loss of OmpF from the translocon. Initial cross-linking studies to delineate this binding site yet further suggest that the site includes residue 64 but does not extend beyond residue 75 (N.G.H. and C.K., unpublished results).

Immunity Protein Is Not Released During Formation of the Colicin Translocon. Enzymatic E colicin cytotoxic domains are inactivated in the producing host through the action of an immunity protein that binds with fM affinity to the nuclease (36). The heterodimeric colicin/Im protein complex is then released into the surrounding medium after lysis of the producing cell, induced by a bacteriocin release lipoprotein. Part of our study addressed the issue of whether the Im protein is dissociated at any point during formation of the colicin translocon. We performed ITC measurements on BtuB ($\pm Ca^{2+}$) binding ColE9 that had been stripped of its immunity protein and found that the thermodynamic parameters were very similar to those of the heterodimeric complex (Table 1). Also, because the isolation of the BtuB·ColE9/Im9·OmpF complex from the bacterial outer membrane was accomplished by Ni-affinity purification utilizing a hexa-histidine tag on Im9, we conclude that immunity proteins are not released during formation of the colicin translocon.

We thank Jeremy Lakey (University of Newcastle upon Tyme, Newcastle, U.K.) for the gift of *E. coli* B^{E}3000 cells; William Cramer (Purdue University, West Lafayette, IN) for the gift of porin-minus *E. coli* TNE012 cells; Chris Law (University of Limerick, Limerick, U.K.) for advice on BtuB purification; Chris Penfold and Bryan Healy (University of Nottingham) for the gift of the clone encoding ColE9$^{S\text{-}S}$; Jerry Thomas and Andrew Leech of the York Technology Facility for assistance with MALDI-MS and ITC measurements; and Dan Walker, Anthony Keeble, and Jennifer Potts (University of York) for critical reading of the manuscript. This work was funded by The Wellcome Trust.

1. Falnes, P. O. & Sandvig, K. (2000) *Curr. Opin. Cell Biol.* **12,** 407–413.
2. James, R., Lazdunski, C. J. & Pattus, F. (1992) *Bacteriocins, Microcins, and Lantibiotics* (Springer, Heidelberg, Germany).
3. Brook, I. (1999) *Crit. Rev. Microbiol.* **25,** 155–172.
4. Kirkup, B. C. & Riley, M. A. (2004) *Nature* **428,** 412–414.
5. Lazdunski, C. J., Bouveret, E., Rigal, A., Journet, L., Lloubès, R. & Bénédetti, H. (1998) *J. Bacteriol.* **180,** 4993–5002.
6. James, R., Penfold, C. N., Moore, G. R. & Kleanthous, C. (2002) *Biochimie* **84,** 381–389.
7. Bradbeer, C., Woodrow, M. L. & Khalifah, L. I. (1976) *J. Bacteriol.* **125,** 1032–1039.
8. Lazzaroni, J. C., Dubuisson, J. F. & Vianney, A. (2002) *Biochimie* **84,** 391–397.
9. Lloubès, R., Cascales, E., Walburger, A., Bouveret, E., Lazdunski, C., Bernadac, A. & Journet, L. (2001) *Res. Microbiol.* **152,** 523–529.
10. Di Masi, D. R., White, J. C., Schnaitman, C. A. & Bradbeer, C. (1973) *J. Bacteriol.* **115,** 506–513.
11. Soelaiman, S., Jakes, K., Wu, N., Li, C. & Shoham, M. (2001) *Mol. Cell* **8,** 1053–1062.
12. Kurisu, G., Zakharov, S. D., Zhalnina, M. V., Bano, S., Eroukova, V. Y., Rokitskaya, T. I., Antonenko, Y. N., Wiener, M. C. & Cramer, W. A. (2003) *Nat. Struct. Biol.* **10,** 948–954.
13. Kleanthous, C., Kühlmann, U. C., Pommer, A. J., Ferguson, N., Radford, S. E., Moore, G. R., James, R. & Hemmings, A. M. (1999) *Nat. Struct. Biol.* **6,** 243–252.
14. Wiener, M., Freymann, D., Ghosh, P. & Stroud, R. M. (1997) *Nature* **385,** 461–464.
15. Penfold, C. N., Healy, B., Housden, N. G., Boetzel, R., Vankemmelbeke, M., Moore, G. R., Kleanthous, C. & James, R. (2004) *J. Bacteriol.* **186,** 4520–4527.
16. Griko, Y. V., Zakharov, S. D. & Cramer, W. A. (2000) *J. Mol. Biol.* **302,** 941–953.
17. Law, C. J., Penfold, C. N., Walker, D. C., Moore, G. R., James, R. & Kleanthous, C. (2003) *FEBS Lett.* **545,** 127–132.
18. Wallis, R., Reilly, A., Rowe, A., Moore, G. R., James, R. & Kleanthous, C. (1992) *Eur. J. Biochem.* **207,** 687–695.
19. Chimento, D. P., Kadner, R. J. & Wiener, M. C. (2003) *J. Mol. Biol.* **332,** 999–1014.
20. Kleanthous, C., Hemmings, A. M., Moore, G. R. & James, R. (1998) *Mol. Microbiol.* **28,** 227–233.
21. Mohanty, A. K., Bishop, C. M., Bishop, T. C., Wimley, W. C. & Wiener, M. C. (2003) *J. Biol. Chem.* **278,** 40953–40958.
22. Chimento, D. P., Mohanty, A. K., Kadner, R. J. & Wiener, M. C. (2003) *Nat. Struct. Biol.* **10,** 394–401.
23. Bouveret, E., Rigal, A., Lazdunski, C. & Bénédetti, H. (1997) *Mol. Microbiol.* **23,** 909–920.
24. Carr, S., Penfold, C. N., Bamford, V., James, R. & Hemmings, A. M. (2000) *Structure (London)* **8,** 57–66.
25. Collins, E. S., Whittaker, S. B., Tozawa, K., MacDonald, C., Boetzel, R., Penfold, C. N., Reilly, A., Clayden, N. J., Osborne, M. J., Hemmings, A. M., *et al.* (2002) *J. Mol. Biol.* **318,** 787–804.
26. Anderluh, G., Hong, Q., Boetzel, R., MacDonald, C., Moore, G. R., Virden, R. & Lakey, J. H. (2003) *J. Biol. Chem.* **278,** 21860–21868.
27. Mock, M. & Pugsley, A. P. (1982) *J. Bacteriol.* **150,** 1069–1076.
28. Schwarz-Linek, U., Pilka, E. S., Pickford, A. R., Kim, J. H., Hook, M., Campbell, I. D. & Potts, J. R. (2004) *J. Biol. Chem.* **279,** 39017–39025.
29. Lacy, E. R., Filippov, I., Lewis, W. S., Otieno, S., Xiao, L., Weiss, S., Hengst, L. & Kriwacki, R. W. (2004) *Nat. Struct. Mol. Biol.* **11,** 358–364.
30. Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N. & Rosenbusch, J. P. (1992) *Nature* **358,** 727–733.
31. Rigal, A., Bouveret, E., Lloubès, R., Lazdunski, C. & Bénédetti, H. (1997) *J. Bacteriol.* **179,** 7274–7279.
32. Fink, A. L. (2005) *Curr. Opin. Struct. Biol.* **15,** 35–41.
33. Dyson, H. J. & Wright, P. E. (2005) *Nat. Rev. Mol. Cell Biol.* **6,** 197–208.
34. Garinot-Schneider, C., Penfold, C. N., Moore, G. R., Kleanthous, C. & James, R. (1997) *Microbiology* **143,** 2931–2938.
35. Zakharov, S. D., Eroukova, V. Y., Rokitskaya, T. I., Zhalnina, M. V., Sharma, O., Loll, P. J., Zgurskaya, H. I., Antonenko, Y. N. & Cramer, W. A. (2004) *Biophys. J.* **87,** 3901–3911.
36. Kleanthous, C. & Walker, D. (2001) *Trends Biochem. Sci.* **26,** 624–631.