Published in final edited form as:

Biochemistry. 2016 September 13; 55(36): 5084–5094. doi:10.1021/acs.biochem.6b00621.

The Colicin E1 TolC-Binding Conformer: Pillar or Pore Function of TolC in Colicin Import?

Stanislav D. Zakharov, Xin S. Wang[†], and William A. Cramer^{*}

Department of Biological Sciences, Purdue University, Hockmeyer Building of Structural Biology, West Lafayette, Indiana 47907, United States

Abstract

The mechanism by which the drug export protein TolC is utilized for import of the cytotoxin colicin E1 across the outer membrane and periplasmic space is addressed. Studies of the initial binding of colicin E1 with TolC, occlusion of membrane-incorporated TolC ion channels, and the structure underlying the colicin–TolC complex were based on the interactions with TolC of individual colicin translocation domain (T-domain) peptides from a set of 19 that span different segments of the T-domain. These studies led to identification of a short 20-residue segment 101–120, a “TolC box”, located near the center of the colicin T-domain, which is necessary for binding of colicin to TolC. Omission of this segment eliminated the ability of the T-domain to occlude TolC channels and to co-elute with TolC on a size-exclusion column. Far-ultraviolet circular dichroism spectral and thermal stability analysis of the structure of T-domain peptides implies (i) a helical hairpin conformation of the T-domain, (ii) the overlap of the TolC-binding site with a hinge of the helical hairpin, and (iii) a TolC-dependent stage of colicin import in which a central segment of the T-domain in a helical hairpin conformation binds to the TolC entry port following initial binding to the BtuB receptor. These studies provide the first structure-based information about the interaction of colicin E1 with the unique TolC protein. The model inferred for binding of the T-domain to TolC implies reservations about the traditional model for colicin import in which TolC functions to provide a channel for translocation of the colicin in an unfolded state across the bacterial outer membrane and a large part of the periplasmic space.

Graphical abstract

^{*}Corresponding Author: waclab@purdue.edu. Phone: 765-494-4956.

[†]X.S.W.: Health Science Center, Texas Tech University, Lubbock, TX 74409.

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bio-chem.6b00621.

Figure S1 shows the empirical dependence of helical content on the ratio of amplitudes of the CD signal at 222 and 208 nm, CD₂₂₂/CD₂₀₈. The far-UV CD spectra of T-peptides containing aromatic side chains, the helical content of which was determined using the DichroWeb package of programs, were used for this plot. The far-UV CD spectra of naturally disordered proteins α -synuclein and the T1–83 peptide of colicin E3 were used to plot data for zero helical content (PDF)

Little is known about the detailed molecular mechanisms by which macromolecules are exported,^{1,2} and disease-causing cytotoxins are imported, across the cell envelope of Gram-negative bacteria.³ Among the cytotoxins, the problem has been addressed in greatest detail for the colicins that enter *Escherichia coli*.^{4–9} The functions that underlie colicin activity are encoded in separate structural domains, C-terminal (lethal function), R (receptor binding), and N-terminal T (translocation) (Figure 1A). Ribbon diagrams that represent structures of an endoribonucleolytic colicin, E3¹⁰ (Figure 1B), the channel-forming colicins Ia,¹¹ B,¹² and N¹³ (Figure 1C–E), and the channel-forming domains of colicins E1¹⁴ and A¹⁵ (Figure 1F,G) are shown, along with the outer membrane/trans-periplasm protein, TolC¹⁶ (Figure 1H). It can be noted that because of the coiled-coil structure of the R-domain, the T- and C-domains in colicins E3 and Ia are proximal (Figure 1B,C), whereas they are on opposite sides of the structures of colicins N and B, connected through a single long helix (Figure 1D,E), which suggests different import mechanisms of the latter colicins.

The import of the 522-residue colicin E1,^{5,17} for which a structure of the C-terminal channel-forming domain is available (Figure 1F),¹⁴ is the subject of this study. Colicin E1 exerts its cytotoxic effect by forming ion permeable channels of sufficient conductance in the cytoplasmic membrane, approximately 10^6 ions/s per channel,⁵ to dissipate the transmembrane proton electrochemical potential gradient, resulting in membrane deenergization and cell death. For cell entry, colicin E1 uniquely uses the outer membrane periplasmic membrane protein, TolC (Figure 1H), which is embedded in the outer membrane as a 12-stranded β -barrel connected to 12 α -helical strands that form a tunnel spanning a major fraction (~ 100 Å) of the periplasmic space.^{16,18}

Passage of colicins across the outer membrane involves initial high-affinity binding to an outer membrane protein whose normal function is to transport cell metabolites such as

siderophore-bound iron^{19,20} or vitamins. Import of colicin E1 is initiated by binding to the cobalamin translocator BtuB.²¹ The structure of BtuB has been determined to high resolution in the absence^{22,23} and presence of the receptor-binding domain for colicin E3²⁴ and E2.²⁵

TolC is a component of the bacterial drug and hemolysin efflux complexes, and a partner in several classes of tripartite multidrug efflux machines.^{2,26–28} It has been proposed that passage of colicin E1 through the outer membrane and periplasmic space results from directed diffusion or translocation through the TolC pore/channel.^{9,29} An alternate view is suggested in the study presented here.

The relatively open pore of wild-type TolC in the β -barrel tapers to a narrow constrictive opening that is 3–4 Å in diameter at its terminal opening in the periplasm and would have to open to allow outward passage of the colicin polypeptide. This iris-like terminal conformation change would be similar to that which has been proposed for the export through TolC of drugs and macrolides.^{16,28,30} Crystallographic and mutagenic studies suggest that interactions with one or more of its periplasmic partners, such as the AcrA–AcrB complex, drive TolC into a more open configuration,^{31,32} to allow the passage of drugs through its channel. However, analysis of the Keio collection of the bacterial genome indicates that the Acr and other drug export proteins are not involved in colicin E1 import.³³

The T-domain of colicin E1 occludes TolC channels incorporated into planar lipid bilayer membranes,³⁴ as also described for the T-domains of the nucleolytic colicins E3 and E9, bound to their OmpF translocator.^{35,36} A limit on the extent of penetration of colicin E1 into the TolC pore was inferred from mutations of TolC residue Gly43, which faces the inside of the β -barrel domain of TolC and is close to the boundary between the β -barrel and α -helical domains of TolC. Such mutations reduced the killing efficiency of colicin E1, suggesting that passage of the colicin through the channel was obstructed by mutant amino acids with larger side chains at this position.³⁷ However, details of the initial structural interaction of colicin E1 with TolC, and the mechanisms of the proposed translocation of colicin E1 through TolC,^{16,38,39} and consequently into the periplasmic space, are presently not understood.

In this study, occlusion of TolC channels by a series of individual colicin E1 T-domain peptides identified a short segment of no more than 20 residues, a “TolC box”, near the middle of the classically defined colicin “translocation domain”; its presence is necessary for TolC channel occlusion by colicin E1 and co-elution with TolC on a SEC column. This inference is also supported by *in vivo* protection of *E. coli* cells by T-domain peptides against colicin E1 cytotoxicity (K. S. Jakes, unpublished data).⁴⁰ Circular dichroism analysis of the secondary and tertiary structure of T-peptides implies a helical hairpin conformation of the T-domain with the TolC-binding site that includes a hinge of the helical hairpin bound to the TolC entry port.

EXPERIMENTAL PROCEDURES

Peptide Constructs

Colicin E1 T-domain peptides with the C-terminal His₁₀ tag listed in Table 1 as well as synthetic peptide T100–120 (H-EALRHNASRTPSATELAHANN-OH) were provided by K. S. Jakes. Except for synthetic peptide T100–120, these peptides have an additional C-terminal 21-residue sequence containing a thrombin cleavage site and His₁₀ tag, ELALVPRGSSA(H)₁₀, which resulted from cloning in the pET52b plasmid.

Peptides T1–40, T1–81, T1–190, T41–190, T81–141, and T141–190 have the C-terminal His₈ tag, LE(H)₈, that resulted from cloning in the pET41b vector.

Protein Purification

E. coli strain BL21(DE3) transformed with the respective plasmid was used for peptide expression under control of an IPTG-inducible T7 polymerase promoter. Cells were grown in Luria-Bertani (LB) medium and purified by nickel chelation chromatography. Peptides were dialyzed in 50 mM sodium phosphate (pH 7.0) using dialysis membranes with 2, 7, or 10 kDa cutoff pores.

The TolC trimer was purified as previously described³⁴ and stored in 0.03% DDM, 20 mM Tris-HCl (pH 7.5), and 0.1 M NaCl.

Cleavage of the His₁₀ Tag from T-Domain Peptides

The C-terminal His tag was removed from T1–190 and T1–140 using thrombin. Peptide digestion was conducted overnight at room temperature, using 2 units of thrombin/mg of peptide. The reactions were terminated with 1 mM PMSF. His₁₀ tag removal was confirmed by SDS–PAGE in 12% polyacrylamide.

TolC Channel Conductance Measurements in Planar Bilayer Lipid Membranes

TolC channel conductance was measured in BLM. Because of the low efficiency of spontaneous incorporation of TolC into planar membranes from the detergent-dissolved state, TolC was initially incorporated into liposomes, and then the proteoliposomes were fused with lipid bilayer membranes.⁴¹ The BLM and liposomes had a 2:3:5 DOPG:DOPC:DOPE lipid composition.

Preparation of Liposomes

Lipids (20 mg) dissolved in chloroform were mixed, and the chloroform was removed by vortexing in a nitrogen stream. To remove traces of chloroform, the tube containing lipids was stored under vacuum for 2–3 h. One milliliter of the buffer solution [5 mM HEPES (pH 7.2) and 50 mM KCl] was added, and the lipid suspension in a glass tube was vortexed under N₂ to prevent lipid oxidation. The lipid suspension was subjected to five freeze–thaw cycles while being subjected to vigorous vortexing. The liposomes (SUV) were formed by sonication until clarification was achieved.

Reconstitution of TolC into Liposomes

Small uni-lamellar vesicles (SUV) were mixed with the detergent *n*-octyl β -D-glucoside (OG); TolC was added to obtain a lipid/TolC mixture with a molar ratio of approximately 1000 and a final OG concentration of 2%. The mixture was incubated on ice (2 h) and dialyzed (40–60 h) at 4 °C with two or three changes of dialysis buffer [10 mM Tris (pH 7.5), 50 mM KCl, and 0.3 M sucrose].

Planar Lipid Bilayer Membranes

These membranes were formed on an aperture (0.25 mm diameter) in a partition separating two 1 mL compartments, using a lipid mixture (10 mg/mL) of DOPG, DOPC, and DOPE (2:3:5 molar ratio) in *n*-decane.³⁴ The aqueous solutions in the *cis* and *trans* compartments contained 0.1 and 0.4 M KCl, respectively, buffered with 5 mM sodium phosphate (pH 7.0). TolC proteoliposomes (0.5–2.0 μ L) were added to the *trans* compartment, and the solutions were stirred until channels appeared. Fusion of liposomes with BLM is driven by an osmotic and salt concentration gradient between *cis* and *trans* compartments and across the liposome membrane.

For channel measurements at symmetric salt concentrations, the *trans* chamber was perfused with 5–10 mL of 0.1 M KCl. Colicin peptides were added to the *cis* or/and *trans* side of the membrane, and solutions in both chambers were stirred for 2 min. The membrane current was measured in voltage-clamp mode with Ag/AgCl electrodes connected through agar bridges with 3 M KCl, using a WARNER BC-525C amplifier (Warner Instruments, Hamden, CT). A transmembrane potential was generated by applying a voltage to the electrode on the *cis* side of the membrane.

Far-UV Circular Dichroism Spectra

CD spectra of colicin E1 peptides in the far-UV range (185–260 nm) were measured in quartz cuvettes (Hellma, optical path lengths of 0.02, 0.1, and 0.2 mm), using a “Chirascan” spectropolarimeter (Applied Photophysics, Ltd., Leatherhead, U.K.) equipped with a Peltier thermal control unit. The buffer was 50 mM sodium phosphate (pH 7.5).

CD spectral amplitudes expressed in mean molar residue ellipticity units were analyzed using the DichroWeb package of Selcon3, Contin, and CDSSt programs and respective sets of reference proteins (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>).⁴²

Because of the absence of aromatic residues in colicin E1 segment 58–190, the helical content of such peptides was estimated through the ratio of amplitudes of the CD spectrum at 222 and 208 nm (CD_{222}/CD_{208}). The helical content and the parameters, CD_{222}/CD_{208} , of peptides containing aromatic residues were used to plot this dependence (Figure S1). Far-UV CD spectra of colicin E3 peptide T1–83 and of the α -synuclein polypeptide⁴³ were used to plot CD_{222}/CD_{208} for proteins lacking ordered secondary structure (helical content, 0%).

Thermal Melting

Thermal melting of peptides was measured in a 1 cm quartz cuvette with magnetic stirring in 50 mM potassium phosphate (pH 7.0) as a function of the amplitude of the 222 nm CD signal versus temperature, with a rate of temperature increase of 0.5 °C/min.

Co-elution of T-Domain Peptides and TolC on a Superdex 200 Column

TolC and T-domain peptides were mixed in a 1:2 molar ratio and incubated for 20 min, and the mixture was eluted through a Superdex 200 column (10 mm × 300 mm) in 10 mM Tris (pH 7.5), 0.1 M NaCl, and 0.03% DDM. TolC alone and respective peptides alone were chromatographed separately. Co-elution of TolC and peptides was detected by the TolC peak shift; the presence of peptides in the TolC peak fractions was detected by SDS–PAGE on a 12% polyacrylamide gel.

RESULTS

Localization of the TolC-Binding Site in the Colicin E1 T-Domain

In contrast to other group E colicins whose interaction with the putative outer membrane translocator is initiated by binding of the disordered N-terminus to the OmpF porin,^{35,36} it has been shown that the disordered glycine-rich N-terminal segment 1–40 of colicin E1 does not interact with TolC.⁴⁴ Neither T1–40 nor T1–81, each of which is predominately disordered (Table 1), interacted with TolC, whereas peptides 1–190 and 41–190 showed strong binding, as revealed by co-elution with TolC on a size-exclusion column,⁴⁴ implying that the colicin E1 TolC-binding site is located in T-domain segment 81–190. Here, an extended set of peptides was probed for interaction with TolC to more precisely determine the TolC-binding site in the colicin E1 T-domain.

Co-elution of T-Peptides with TolC—The TolC trimer and T-peptides were mixed at a 1:2 molar ratio and eluted through a Superdex 200 column. Co-elution of TolC and T-peptides was detected by the shift of the TolC peak and a significant decrease in the intensity of the T-peptide peak (Figure 2A), as well as by the presence of the T-peptide in TolC fractions analyzed by SDS–PAGE (Figure 2B). Co-elution was detected for peptides T1–190, T1–140, T1–120, T41–190, and T57–190, but not for peptides T1–100, T1–81, T1–40, and T1–190 (100–120) (Table 1). Mixing of TolC with peptides T57–120 and T57–140 caused severe protein precipitation, implying interaction between TolC and these peptides.

Occlusion of TolC Ion-Conductive Channels by Particular T-Domain Peptides

—TolC was reconstituted into liposomes, and TolC proteoliposomes were fused with BLM using a transmembrane salt gradient (0.1 and 0.4 M KCl in *cis* and *trans* compartments). After the appearance of ion-conductive channels in BLM, the salt concentration in the *trans* compartment was reduced by perfusion with 0.1 M KCl, to conduct measurements under symmetric salt conditions.

Addition of any T-peptide that contained colicin segment 100–120 resulted in a significant and irreversible decrease in transmembrane current (Table 1), as shown by addition of T100–140 to a final concentration of 2 µg/mL on both sides of the BLM (Figure 3A, blue and red

traces in the absence and presence of T-peptide, respectively). Addition of peptide T1–100 did not decrease the transmembrane current [Figure 3B, blue and green traces for before and after addition of a relatively high concentration of T1–100 (10 $\mu\text{g}/\text{mL}$), respectively]. Subsequent addition of T41–190 to a final concentration of 3 $\mu\text{g}/\text{mL}$ significantly decreased the transmembrane conductance of the TolC channel (Figure 3B, red trace).

Particularly notable is the absence of an interaction between TolC and the T-domain peptide T1–190 (100–120). Deletion from T1–190 of the 100–120 segment abolished the ability of the peptide to occlude TolC channels and to co-elute with TolC from an SEC column. Analysis of the *in vitro* interaction pattern of the whole set of analyzed peptides implies that the segment spanning residues 100–120 comprises the essential TolC-binding site, the “TolC box” of colicin E1 (Table 1). There is complete correspondence of the ability of the T-peptides to protect *E. coli* cells against colicin E1 cytotoxicity (K. S. Jakes, unpublished data)⁴⁰ and occlusion of TolC channels (Table 1), implying that binding *in vivo* of T-peptides to TolC containing the intact TolC-binding site prevents the effective interaction of colicin E1 with TolC.

It was determined that a synthetic peptide consisting of residues 100–120 without a His tag did not occlude TolC channels (Table 1). This might imply that (i) effective interaction with TolC requires sequences adjacent to the 100–120 domain in the colicin T-domain and/or (ii) a particular tertiary structure of segment 100–120, such as a hinge of a helical hairpin that is able to insert deeply enough into the TolC β -barrel, is required for effective TolC binding. It is noted that, unlike the partially helical structure of T1–120 and longer peptides (Table 1), the synthetic peptide 100–120 is totally disordered. We concluded that residues 100–120 contain the essential binding epitope of E1 for its TolC translocator and define it as the “TolC box”. As the synthetic T100–120 peptide, unlike all tested T-domain peptides, did not contain a C-terminal histidine tag, the His tag was removed from both T1–190 and T41–190 by thrombin digestion, without any effect on the ability of these peptides to occlude TolC channels.

Estimate of Binding Affinity—Peptide T57–140 efficiently occluded TolC channels at concentrations of 0.2 μM in the bathing solution ($I = 0.1 \text{ M}$, pH 7.5) but did not occlude at a concentration of 40 nM, implying that the colicin E1 T-domain has a binding affinity for TolC of $\approx 10^{-7} \text{ M}$.

Role of Electrostatic Interactions in TolC Binding of T-Domain Peptides—At neutral pH, the N- and C-terminal segments of the colicin E1 T-domain are negatively charged, with a pI of ~ 5 for segments T1–40 and T141–190, and a central domain that is positively charged, with a pI of 9.6 for segment T81–120 (Figure 4A). Segment 100–120 contains two Arg residues, R103 and R108. These residues were mutated to Gln side chains to determine whether Arg side chains are responsible for interaction with TolC, whose interior is electronegative.⁴⁵ Whereas single mutations R103Q and R108Q in the T57–140 peptide had a small effect on the rate of channel occlusion, the double mutant R103Q/R108Q significantly decreased the rate of TolC occlusion, although the final extent of occlusion was the same as that caused by the wild-type T57–140 peptide (Figure 4B).

Structural Prediction for the Translocation Domain of Colicin E1

It was shown previously that all three domains of colicin E1 are predominantly α -helical, with helical contents of 70, 80, and 67% for the T-, R-, and C-domains, respectively.^{8,32,34,35} The C-terminal cytotoxic domain forms a tightly packed nine-helix globule in aqueous solution (Figure 1F, PDB entry 2I88, 2.5 Å¹⁴).

Ordered and Disordered Secondary Structure of Colicin E1 T-Domain Peptides

—The far-UV CD spectra of T-peptides dissolved in 50 mM sodium phosphate were measured in the spectral range of 185–260 nm (Figure 5). The secondary structure content of the different segments of the T-domain was determined using DichroWeb CD spectral analysis.⁴³ This analysis depends on precise measurement of the protein concentration. The T-domain has three tyrosines (Y7, Y8, and Y14) and one tryptophan (W57). The helical content of peptides that lack aromatic residues (T81–140, T100–140, and T100–190) was inferred from the ratio of amplitudes of the CD signals at 222 and 208 nm (Q_{222}/Q_{208}). This ratio increases with an increase in helical content.⁴⁶ A calibration curve for the dependence of Q_{222}/Q_{208} on the fractional helical content was created using peptides containing aromatic side chains (Figure S1). Data on the fractional helical content and the total number of residues in a helical conformation (Table 1) are derived from far-UV CD spectra, taking into account the presence of the C-terminal 21- or 10-residue His tag, assuming that the tags are unordered and that the T-domain does not contain β -structure. DichroWeb analysis of CD spectra of all peptides (Figure 5) confirmed the absence of β -structure in the colicin E1 T-domain.

The N-terminal translocation domain (residues 1–190) has an unordered glycine-rich N-terminal segment that extends at least to residue 40 and a predominantly α -helical segment of residues 41–190.⁴⁴ However, peptide 1–81 has a low helical content (Table 1), implying that significant disordering is present in segment 41–81. An increase in peptide length from 81 to 140 residues results in a respective increase in helical content in peptides 1–100, 1–120, and 1–140. However, a low level of disordering is present in segments 81–100 and 101–120 [increase in helicity, 17 and 16 residues, respectively (Table 1)], whereas segment 121–140 is entirely helical. A further 50-residue increase in peptide length in peptide 1–190 shows an increase in helical content of >80 residues, implying that (i) segment 141–190 is entirely helical and (ii) the presence of this segment induces helix formation in segment 41–81 of the T-domain. This inference is confirmed by the significantly lower content of unordered secondary structure in T1–190 compared with those of peptides 1–81, 1–100, 1–120, and 1–140 [approximately 40 and 70 residues, respectively (Table 1)].

Induced Helicity and Interhelix Interactions in the T-Domain—The 154 residue helical content of the T-domain [T1–190 (Table 1)] is significantly larger than a sum of the helical content of the components of this domain (T1–140 and T141–190, containing 68 + 45 = 113 residues). This increment is most readily explained by interhelix interactions in a folded looped structure of the T-domain as shown for the colicin Ia structure (Figure 1C). Thermal melting analysis confirms interhelix interactions in the T-domain, which result in highly cooperative melting of peptides T1–190 and T41–190 (Figure 6, blue and red traces, respectively), whereas the melting of peptides T1–140, T141–190, and T100–190 is not

cooperative (Figure 6, green, pink, and orange traces, respectively). It is inferred that the putative helix–helix interaction must occur between the helix in segment 141–190 and that in segment 41–100. Therefore, a segment near the middle of peptide T41–190 is inferred to have a hingelike conformation that connects the two interacting helical segments. Segment 101–120 at the interface between the two domains of opposite electrical charge (Figure 4A) is likely to possess the hinge function, as summarized below (Discussion).

The interhelix interactions in the T-domain have a partly electrostatic nature, as inferred from their dependence on salt concentration (Figure 6, inset). An increase in salt concentration shifts the peptide melting to a lower temperature but does not eliminate the cooperativity of melting, implying that nonelectrostatic forces play a significant role in the formation of T-domain tertiary structure.

TolC Binding Does Not Cause a Decrease in the Helical Content of T-Peptides

—To determine whether binding of the T-domain to the TolC trimer causes changes in its secondary structure, far-UV CD spectra of peptides T1–140, T1–190, and T41–190, the TolC trimer, and TolC in complex of one of these peptides at a 1:1 molar ratio were recorded. The spectra of the peptides alone and in complex with TolC were compared in difference CD spectra, “TolC/T-peptide minus TolC” (Table 2). Assuming that the helical content of TolC did not change upon binding with T-peptides, the results imply that the helical content of T-peptides in complex with TolC does not decrease; i.e., the peptides do not unfold. Moreover, for peptide T1–140, an increase in helicity from 37 to 59% was detected (Table 2). Therefore, the helical hairpin structure of the TolC-binding conformer centered around residues 100–120 is preserved in the TolC-bound state of the T-domain.

DISCUSSION

The TolC-Interactive Domain of Colicin E1, the “TolC Box”

By creating a series of increasingly shorter peptides corresponding to different segments of the 190-residue T-domain of colicin E1, we have identified a sequence of no more than 20 residues, residues 101–120, that is required for the interaction of the T-domain with the outer membrane/periplasmic protein, TolC. Peptides that contain that sequence occlude TolC channels, and this function is abolished by deletion of that segment. The effective dissociation constant ($K_d \approx 10^{-7}$ M) for the binding of these peptides with TolC reflects an affinity 10–100-fold stronger than that estimated for the binding of a colicin E9 translocation domain peptide to the pore domain of the OmpF porin,³⁵ proposed to be used for passage of E9 into the periplasm.⁴⁷

Tertiary Structure Prediction for T-Domains of Colicin E1

An explanation for the inability of the synthetic peptide T100–120, which lacks ordered secondary structure, to occlude TolC channels, and to protect *E. coli* cells (K. S. Jakes, unpublished data), resides in the structure of this segment in the colicin E1 T-domain.

N-Terminal segment 1–40, containing two Pro residues (P13 and P29) and 12 Gly residues, is unordered. The 150 residues in helices of T1–190 inferred from far-UV CD analysis (Figure 5 and Table 1) imply that not more than two helices are present in the T-domain. The

interhelix interaction in the T-domain inferred from thermal stability analysis (Figure 6) implies a helical hairpin conformation in segment 41–190. Analogy with another similar channel-forming colicin, colicin Ia, for which the crystal structure is known,¹¹ shows the T-domain to be characterized by a helical hairpin that occupies most of this domain, with the turn or hinge approximately in the middle of the hairpin (Figure 1C, T-domain colored blue).

The hingelike structure in the colicin E1 T-domain is inferred to be located in or near segment 101–120 for the following reasons. (a) The putative hinge in segment 101–120 is close to the center of peptide T41–190. (b) The only proline residue, P110, in segment 41–190, which will facilitate a turn, is in the center of segment 101–120. (c) The hairpin conformation would be strengthened by electrostatic interactions in the T-domain between the basic and acidic segments, 60–100 and 141–190 [pI values of 9.6 and 5, respectively (Figure 4A)], and confirmed by a dependence of the thermal melting transition on salt concentration for T41–190 (Figure 6, inset). (d) Such a hinge of the hairpin can be accommodated in the TolC portal, whereas peptide T100–120 in a random-coil conformation (Table 1) is too large to insert into the TolC β -barrel and therefore does not occlude the TolC channels (Table 1) and does not protect *E. coli* cells (K. S. Jakes, unpublished data).

Given the positively charged nature of the TolC box and adjacent sequences, the possibility that the preponderance of basic residues within segment 101–120 (Figure 4A), or a similar closely neighboring segment, helps position the TolC box sequence within the TolC barrel was considered. It was shown, however, that mutating the two positively charged residues (Arg103 and Arg108) within the TolC box decreases the rate of TolC occlusion but does not eliminate *in vivo* protection against colicin E1 cytotoxicity (K. S. Jakes, unpublished data) or TolC occlusion of TolC ion channels (Figure 4B).

A Model for Interaction of the Colicin E1 T-Domain with TolC

A model derived from this study for the initial binding of the colicin E1 T-domain to TolC is depicted (Figure 7A). The colicin “TolC box”, segment 101–120 (red), is positioned in TolC as a hingelike structure between adjacent interacting helices on its N- and C-terminal sides. It is inferred to be inserted into TolC beyond its β -barrel, at least into the upper part of the helical “tunnel”, because mutation of residue G43 (pink) at the bottom of the TolC β -barrel to a residue with larger side chains decreased colicin E1 cytotoxicity by as much as 1000-fold.³⁷

For this model, a ribbon diagram of the colicin Ia T-domain was used (residues 23–171, PDB entry 1CII, 3.0 Å⁴), which closely resembles the colicin E1 T-domain, i.e., a helical hairpin consisting of two long helices (55 and 43 residues) with N-terminal segment 23–65 lacking ordered secondary structure.

Implications for Colicin E1 Import

Multiple interactions of the colicins with a number of proteins in the target bacteria are required for their cytotoxic domains to ultimately reach their targets in the cell. The first of those interactions is the tight binding of the R-domain to the outer membrane receptor, BtuB in the case of colicin E1 (Figure 7B). The initial binding to BtuB facilitates the search⁴⁸ in the outer membrane by the colicin T-domain for the secondary receptor (or translocator).

Deep insertion of the helical hairpin of the T-domain into TolC must cause stretching, bending, and unfolding of the N-terminal helix of the R-domain, i.e., some extent of coiled-coil unfolding (Figure 7B), thus increasing the degree of freedom of the C-terminal cytotoxic domain.

Mechanism of Import of Colicin across the Outer Membrane. Import of Colicins E3 and E9—The ultimate consequence of import of colicin across the outer membrane is delivery of its cytotoxic domain to the cytoplasmic membrane in the case of the pore-forming colicins (E1, Ia, N, and B) or into the cytoplasm for the nuclease colicins (E2–E9 and D). On the basis of the crystal structures of the N-terminal domain of the nuclease colicins E3³⁶ and E9³⁵ inserted into OmpF, and mass spectroscopic analysis of a colicin E9–BtuB–OmpF–TolB complex,⁴⁷ it has been inferred that the entire colicin molecule, including the R-domain tightly bound to BtuB²⁴ and the C-terminal cytotoxic domain, is translocated through OmpF into the periplasm. It can be said that this is an imposing task, sterically and energetically. Such a “mass transit” model for translocation of the entire colicin E1 molecule through the TolC pore has the same problem.

In the case of colicin E3, an alternative model has been proposed on the basis of experimentation. The C-domain of colicin E3, which forms a tight complex with the Imm protein in the intact colicin (Figure 1B) and in this state is not able to occlude the OmpF pore, attains this ability after Imm removal.⁴⁹ Removal of Imm results in unfolding of the C-domain.⁴⁹ Therefore, it was proposed that after binding of the colicin E3 R-domain with BtuB, the T-domain recruits the OmpF trimer by insertion of its N-terminus into one pore and anchoring by binding with the β -propeller domain of periplasmic protein TolB.⁴⁷ When the fact that the Imm protein in colicin E3 is bound between the T- and C-domains is taken into account (Figure 1B, yellow), simultaneous binding of R- and T-domains to BtuB and OmpF can cause Imm release, C-domain unfolding, and insertion of the C-domain into and threading of the C-domain through a second pore of the recruited OmpF trimer into the periplasm.⁴⁹

Such a detailed model is not applicable to the import of the colicin E1 C-domain, but for the nuclease colicins, it suggests consideration of a translocation mechanism, details presently unknown, that would avoid the energetically imposing translocation of the entire colicin molecule across the outer membrane to accomplish translocation of the distal C-domain.

Alternative Mechanisms for Import of Colicin E1 across the Outer Membrane

—The proposed structure of the T-domain bound to TolC (Figure 7A,B) does not provide an obvious route for insertion of the T-domain through the TolC pore or for the subsequent transfer of the colicin receptor-binding and cytotoxic domains into the intermembrane periplasmic space. The colicin–TolC complex is displayed in Figure 7B based on peptide protection against colicin cytotoxicity (K. S. Jakes, unpublished data),⁴⁰ channel occlusion (Table 1), and circular dichroism studies (Figures 5 and 6), with the rest of the colicin E1 molecule, showing the R-domain bound to the BtuB receptor. In the context of the ultimate translocation of the cytotoxic domain, this structure presents a difficult topological problem. Both the N-terminal 100 residues, which are necessary for colicin cytotoxicity,³⁷ and the C-domain whose depolarizing action on the cytoplasmic membrane is responsible for

cytotoxicity, and which must enter the periplasm, are facing toward the outside of the target cell. With regard to the proposed role of TolA in colicin E1 import,⁵⁰ it is noted that experiments focused on its action *in vivo* imply that its function is unclear.⁵¹

An additional formidable problem for translocation through TolC concerns the mechanism by which an extended polypeptide chain can exit the narrow (3.9 Å diameter) iris-like constriction at the periplasmic end of TolC.⁵² During drug or toxin export, the inner membrane/periplasmic partners of TolC provide the energy, via inner membrane proton antiport or ATPase activities, combined with a periplasmic adaptor, to open the iris of TolC.¹⁸ However, none of the usual periplasmic partners of TolC, such as AcrAB, MacAB, and EmrAB, which are involved in *E. coli* efflux pumps, has been implicated in E1 import or intoxication.^{28,33,53,54} The TolC “iris problem” is, in any case, secondary at present because of the “reversed topology” of the colicin E1 molecule bound to TolC that seems to preclude passage of colicin E1 through TolC (Figure 7A,B). Overall, there appears to be no documentation of the actual transport of any macromolecule species through TolC.

From the tightness of binding of the “TolC box”, residues 100–120, to TolC, and the requirement of the N-terminal domain, residues 1–100, for cytotoxicity, it is suggested that the binding of these domains of colicin E1 can utilize TolC as a “pillar” to support the transfer of the cytotoxic domain of the colicin through the outer membrane into the periplasm. Further details of this hypothetical mechanism of utilization of a TolC pillar are presently not understood. However, it is suggested that alternate mechanisms of colicin import should take into account other sectors of the outer membrane, including the LPS.^{55,56} The negative charge of the LPS layer can provide a driving force for direct interaction of the colicin E1 cytotoxic domain (pI 9.0) with the outer membrane of *E. coli*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

These studies were supported by the Henry Koffler Distinguished Professorship and National Institute of General Medical Sciences Grant 038323 (W.A.C.).

Infrastructure support was facilitated by National Institutes of Health Center Grant P30 CA023168. We thank I. Seleznev for contributions to the initial studies of this problem, Prof. K. S. Jakes for providing the set of defined T-domain peptides, *E. coli* strains, plasmids, and helpful discussions, and Drs. S. Saif Hasan and O. Sharma for helpful discussions about particular aspects of the problem.

ABBREVIATIONS

BLM

planar bilayer lipid membranes

CD

circular dichroism

C-, R-, and T-domains

C-terminal cytotoxic, central receptor-binding, and N-terminal translocation domains, respectively

DDM

n-dodecyl β -D-maltopyranoside

DOPC, DOPE, and DOPG

dioleoylphosphatidylcholine, -ethanolamine, and -glycerol, respectively

I

ionic strength

Imm

immunity protein

IPTG

isopropyl β -D-thiogalactopyranoside

LPS

lipopolysaccharide

OG

n-octyl β -D-glucopyranoside

PDB

Protein Data Bank

PMSF

phenylmethanesulfonyl fluoride

SDS-PAGE

sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEC

size-exclusion chromatography

SUV

small unilamellar vesicles

UV

ultraviolet

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Figure 1.

Ribbon diagrams of colicins and a TolC trimer. (A) Domain arrangement of colicins with N-terminal translocation (blue), central receptor-binding (green), and C-terminal cytotoxicity (red) domains. Imm, E3 immunity protein (yellow). (B–G) Ribbon diagrams of colicins E3 (PDB entry 1JGH, 3.0 Å), Ia (PDB entry 1CII, 3.0 Å), B (PDB entry 1RH1, 2.5 Å), and N (PDB entry 1A87, 3.1 Å) and cytotoxic domains of colicins E1 (PDB entry 2I88, 2.5 Å) and A (PDB entry 1COL, 2.4 Å), respectively. (H) Ribbon diagram of the TolC trimer (PDB entry 1EK9, 2.0 Å), forming a 12-stranded β -barrel in the OM membrane and an α -helical ~100 Å extended tunnel in the periplasm.¹⁸ The hydrophobic helical hairpin in the pore-forming cytotoxic domains of colicins Ia, B, N, E1, and A is colored brown.

Figure 2.

Co-elution of colicin E1 T-domain peptides with TolC on a Superdex 200 size-exclusion column. (A) Elution profiles of TolC alone (peak at 10.6 mL, blue), peptide T1–140 alone (peak at 14.7 mL, green), and a mixture of TolC with T1–140 (peak at 10.5 mL, red) in a 1:2 molar ratio. Note the shift in elution of TolC from 10.6 to 10.5 mL when it is mixed with T1–140, as well as the reduction in the height of the T1–140 peak at 14.7 mL in the mixture. The elution buffer consisted of 10 mM Tris (pH 7.5), 0.15 M NaCl, and 0.03% DDM. (B) SDS–PAGE on a 12% polyacrylamide gel of peak fractions eluted from Superdex 200 columns: (1) mixture of TolC and T1–190, (2) T1–190, (3) molecular weight standards, (4) TolC, (5) T1–140, and (6) mixture of TolC and T1–140. Co-elution of peptides with TolC was detected by the presence of a peptide band in TolC fractions (red ovals).

Figure 3.

Occlusion of TolC ion channels by T-domain peptides of colicin E1. (A) Occlusion of TolC channels by peptide T100-140 (red). (B) Lack of TolC occlusion by peptide T1-100 (green) and subsequent occlusion by peptide T1-140 (red). Current records before the addition of peptides are colored blue. The transmembrane voltage was 50 mV. Peptides were added to both sides of the membrane to a final concentration of 2 $\mu\text{g/mL}$. The bathing solution consisted of 0.1 M KCl and 5 mM Tris-HCl (pH 7.5). The *trans* side is defined as the zero-voltage side. TolC proteoliposomes were added to the *trans* side, containing 0.4 M KCl. After the appearance of channels, the *trans* side was perfused with 0.1 M KCl to create symmetric salt conditions. Occlusion occurred during incubation in the absence of an applied voltage.

Figure 4. Probing the role of basic residues in colicin E1 segment 100–120 in the occlusion of TolC. (A) Charge distribution in the colicin E1 T- and R-domains. The pI was calculated over a 20-residue interval (purple squares). Segment 101–120 is basic (pI 9.65). The pI of segments was calculated using ExPASy.⁵⁷ (B) Effect of Arg/Gln mutation at position 103 and/or position 108 on the time course of TolC occlusion by peptide T57–140 added to a final concentration of 3 $\mu\text{g/mL}$.

Figure 5.

Far-UV CD spectra of colicin E1 T-peptides. Spectra of T-domain peptides T41–120 (blue solid line), T41–140 (green solid line), T41–190 (red solid line), T57–120 (cyan dashed line), T57–140 (dark-green dashed line), and T57–190 (pink dashed line) in main panel and spectra of T1–100 (green line), T1–120 (pink dashed line), T1–140 (blue line), and T1–190 (red line) in the inset were recorded in the spectral range of 185–260 nm, using cuvettes with optical path lengths of 0.02, 0.1, and 0.2 mm. The buffer was 50 mM sodium phosphate (pH 7.0).

Figure 6.

Thermal melting analysis of T-domain peptides reveals strong interaction between C- and N-terminal helices of the T-domain in segments 120–190 and 41–100, respectively. (A) Thermal melting of peptides T1–140 (green), T1–190 (blue), T41–190 (red), T100–190 (orange), T141–190 (pink), and T1–19 was measured at 222 nm as the temperature dependence of the CD signal amplitude in a quartz cuvette (path length, 1 cm), with magnetic stirring. The rate of temperature increase was 0.5 °C/min. The buffer consisted of 0.1 M NaCl and 5 mM potassium phosphate (pH 7.0). The inset shows that the increase in salt concentration decreases the thermal stability of peptide T41–190, implying that T-domain interhelix interaction is partly electrostatic. Thermal melting in 10 mM (blue), 0.1 M (green), 0.4 M (brown), and 1.0 M (red) NaCl with 10 mM Tris-HCl (pH 7.5).

Figure 7.

Model for interaction of the translocation domain of colicin E1 with TolC. (A) The colicin E1 T-domain helical hairpin is inserted into TolC. TolC is colored gray. Residue Gly43 from the homotrimer at the bottom of the β -barrel is colored pink and shown in space-filling mode. The helical hairpin consisting of 51- and 53-residue helices is colored brown with the tip of the hairpin (TolC box) colored red. The atom coordinates of the colicin Ia T-domain, segment 23–172, were used for this model. Note that unordered segment 23–65 packed along the helical hairpin is colored blue (PDB entry 1CII). (B) Complex that initiates colicin E1 translocation, showing the R-domain bound to the outer membrane receptor, BtuB, and the T-domain inserted into TolC. Crystal structures of BtuB with the bound R-domain of colicin E3 (PDB entry 1UJW), the C-domain of colicin E1 (PDB entry 2I88), TolC trimer (PDB entry 1EK9), and the helical hairpin from the colicin Ia T-domain (segment 66–175) were used to depict the route of import of colicin E1 through the outer membrane. The TolQRA complex plays an essential role in colicin E1 translocation. AcrAB, a prominent periplasmic binding partner of TolC,²⁸ is shown, although there is no evidence of its involvement in colicin E1 uptake.

Table 1

Summary of Helical Content and Interaction of T-Domain Peptides of Colicin E1 with TolC: (i) Occlusion of TolC Channels, (ii) Co-elution on a Size-Exclusion Column, and (iii) Secondary Structure Content of Peptides Determined by Far-UV CD Analysis^a

peptide	TolC occlusion ^d	TolC co-elution ^d	helical content (%)	no. of residues	
				helix	unordered ^g
1–190 ^b	+	+	73 ± 3	154 ± 6	36
1–190 ^c	+	+	75 ± 4	150 ± 8	40
1–190 ^b (100–120)	–	–	66 ± 5	125 ± 10	44
1–140 ^b	+	+	42 ± 3	68 ± 5	72
1–120 ^b	+	+	33 ± 8	46 ± 12	74
1–100 ^b	–	–	25 ± 3	30 ± 4	70
1–81 ^c	–	–	14 ± 2	13 ± 2	69
1–40 ^c	–	–	0	0	40
41–190 ^b	+	+	76 ± 5	130 ± 9	20
41–190 ^c	+	+	80 ± 3	128 ± 5	22
41–140 ^b	+	nd	54 ± 4	65 ± 5	35
41–120 ^b	+	nd	29 ± 2	29 ± 2	51
57–190 ^b	+	nd	77 ± 4	119 ± 7	15
57–140 ^b	+	ppt	51 ± 3	54 ± 4	30
57–120 ^b	+	ppt	52 ± 4	44 ± 4	20
81–140 ^b	+	nd	43 ± 4	35 ± 4 ^f	25
81–141 ^c	+	nd	23 ± 2	17 ± 2 ^f	44
100–190 ^b	+	nd	71 ± 5	79 ± 6 ^f	12
100–140 ^b	+	nd	42 ± 4	26 ± 3 ^f	15
139–190 ^b	–	nd	75 ± 4	45 ± 3 ^f	7
141–190 ^c	–	nd	75 ± 2	45 ± 2 ^f	5
100–120 ^e	–	nd	0	0	21

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^gExcept as noted, all peptides had His tags.

^hA 21-residue C-terminal His10 tag with a thrombin cleavage site.

^cAn uncleavable C-terminal LeuGluHis8 tag.

^dA plus or minus sign indicates the presence or failure of ToIC occlusion by peptide, respectively, or co-elution with ToIC. nd, not determined; ppt, precipitation formed upon addition of peptide to ToIC.

^ePeptide 100–120 was chemically synthesized without a His tag.

^fBecause of the large uncertainty in the determination of the concentration of peptides lacking aromatic residues, the helical content was determined using a calibration plot showing the correlation between the helical content and the ratio of amplitudes of CD signals at 222 and 208 nm (Figure S1).

^gThe number of residues in the unordered conformation was determined by subtraction of the number of residues in helices from the total number in the peptide excluding tags.

Table 2Helical Content of T-Peptides Does Not Decrease upon Binding of TolC^a

peptide	helical content (%)	
	peptide alone	TolC-bound peptide
T1-190	73 ± 6	77 ± 4
T1-140	37 ± 5	59 ± 8
T41-190	80 ± 8	83 ± 7

^aFar-UV CD spectra (185–260 nm) of 1 μ M T-peptides, TolC trimer, and their complex at a 1:1 molar ratio were measured in cuvettes with optical path lengths of 0.1, 0.2, and 1.0 mm. Spectra of peptides and difference spectra of TolC-bound peptides (“TolC/T-peptide” – “TolC”) expressed in mean molar residue ellipticity units were analyzed using Selcon3, Contin, and K2D programs on the DichroWeb Internet site.⁴² The buffer consisted of 0.1 M sodium phosphate (pH 7.0) and 0.03% DDM.