Pathways of colicin import: utilization of BtuB, OmpF porin and the TolC drug-export protein

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Abstract

Pathway I. Group A nuclease colicins parasitize and bind tightly (Kd < 10^-9 M) to the vitamin B12 receptor on which they diffuse laterally in the OM (outer membrane) and use their long (>100 Å; 1 Å = 0.1 nm) receptor-binding domain as a ‘fishing pole’ to locate the OmpF porin channel for translocation. Crystal structures of OmpF imply that a disordered N-terminal segment of the colicin T-domain initiates insertion. Pathway II. Colicin N does not possess a ‘fishing pole’ receptor-binding domain. Instead, it uses Omp (outer membrane protein) for reception and translocation, processes in which LPS (lipopolysaccharide) may also serve. Keio collection experiments defined the LPS core that is used. Pathway III. Colicin E1 utilizes the drug-export protein TolC for import. CD spectra and thermal-melting analysis predict: (i) N-terminal translocation (T) and central receptor (BtuB)-binding (R) domains are predominantly α-helical; and (ii) helical coiled-coil conformation of the R-domain is similar to that of colicins E3 and Ia. Recombinant colicin peptides spanning the N-terminal translocation domain defined TolC-binding site(s). The N-terminal 40-residue segment lacks the ordered secondary structure. Peptide 41-190 is helical (78%), co-elutes with TolC and occluded TolC channels. Driven by a trans-negative potential, peptides 82-140 and 141-190 occluded TolC channels. The use of TolC for colicin E1 import implies that the interaction of this colicin with the other Tol proteins does not occur in the periplasmic space, but rather through Tol domains in the cytoplasmic membrane, thus explaining colicin E1 cytotoxicity towards a strain in which a 234 residue periplasmic TolA segment is deleted.

Introduction

An understanding of the molecular mechanism of colicin import would contribute not only to elucidation of the biochemical and biophysical events that are unique to the mechanism of colicin cytotoxicity [1–9], but also to an understanding of fundamental aspects of protein insertion into and translocation across membranes [10]. Because the import of colicins from the extracellular medium to their intracellular sites of action involves translocation across the two membranes, outer and inner/cytoplasmic, to the cytoplasm (RNase and DNase colicins) or into the inner membrane (depolarizing channel formers), their translocation mechanism is among the most complicated. Multiple mechanisms of colicin translocation across the Escherichia coli OM (outer membrane) exist. Three modes are discussed in this space for colicins that act as: (i) an intracellular nuclease, for example, colicins E2, E3 and E9, and channel formers that have two different mechanisms of insertion and passage through the OM, (ii) colicin N, and (iii) colicin E1.

Structure–function analysis of colicins; working concepts

(i) Colicins, and as bacteriophages, parasitize the Omps (outer membrane proteins) used for the uptake of small metabolites (e.g. metals, sugars and vitamins). (ii) There is a large difference in the surface density of the Omps used for primary binding (e.g. BtuB, approximately a few hundred copies/cell; OmpF porin, ~10^4 copies/cell [11]). (iii) The C-terminal, middle and N-terminal domains of colicins, although differing in relative lengths, specify cytotoxic activity (C-domain), high-affinity binding (Kd < 10^-9 nM) binding to initial receptor (R-domain), and weaker binding and translocation (T-domain) through the same or a different Omp receptor. (iv) On the basis of a limited number of examples, an extended (≥100 Å; 1 Å = 0.1 nm) coiled-coil R-domain, found for example in the structures of nuclease colicins E2 [12] and E3 [13,14] or the ion channel-forming colicin Ia [15], is associated with utilization of two receptors for colicin import, one for binding and one for translocation [16].

Crystal structures of C-terminal activity domains have also been obtained for the nuclease colicins E2 [17], E7 [18] and E9 [19], and the channel-forming colicins A [20] and E1 [21]. The crystal structure of the DNase colicin E2 is assumed to be similar to that of the E3 colicin [14], for which a crystal structure has been obtained, as well as a complex...
of its extended coiled-coil R-domain with its Omp receptor, the vitamin B<sub>12</sub> receptor BtuB, which is very similar to the structure of a complex obtained with BtuB and the R-domain of colicin E3 [13]. As discussed below, the present concept for OM import of colicin N, which does not possess a coiled-coil central R-domain and an N-terminal T-domain that is predominantly helical and not disordered [22], is that it uses a single Omp for its import [23,24]. The crystal structure of colicin B [25], which is known to utilize the FepA iron-chelate receptor [26], qualitatively resembles that of colicin N.

**Origin of the fishing pole model**

Structures obtained of a complex of the R-domain of colicin E3 [13] and E2 [12] and BtuB showed the colicin R-domain to be bound obliquely in the ‘Venus Flytrap’ network of exterior loops at an angle of 45° to the putative membrane plane. Thus the lateral extent of this domain over the membrane plane is approximately 80 Å. A geometrically similar structure was subsequently obtained for the R-domain of colicin Ia and its Cir receptor [27].

**Other crystal structures of Omps used for colicin import**

Including siderophores such as FepA [26], Omps relevant to colicin import have been discussed previously [28,29]. A 1.59 Å structure has been obtained of the OmpF porin trimer [without LPS (lipopolysaccharide)] [30], which historically, was the first integral membrane protein to be meaningfully crystallized [31]. At somewhat lower resolution, a complex of OmpF with a 7–8- [31] or 15-residue [32] segment of the N-terminal disordered domain of colicin E3 or E9 respectively was crystallized. It is noted that, before cocrystallization of OmpF with the N-terminal disordered domain of colicin E3, it was shown that colicin E3 or its 83-residue N-terminal domain occluded OmpF channel currents in planar bilayer experiments, the former, but not the latter, requiring a transmembrane potential [30,33]. It was also shown using SEC (size-exclusion chromatography) that a complex of larger and appropriate molecular mass had been formed [30,33]. A concept inferred from the structure studies of the complexes of the N-terminal disordered domain of colicins E3 [30] and E9 [32] is that the translocated colicin domain is disordered [an IUTD (intrinsically unstructured translocation domain)] [32]). An initial reservation to this general concept is that translocation of the C-terminal activity domain would require that it be unfolded, as would be the entire colicin if it is translocated (for an alternative mode, see discussion below of transfer through TolC). The primary receptor, BtuB, for the group A colicins, including E2, E3 and E9, has been solved to 1.95 Å by crystallization in the lipid cubic phase, ‘in meso’ [34], and to 2.00 Å in detergent, ‘in surfato’. It is of interest that BtuB is the only example of an integral membrane protein solved to comparably high resolution ‘in meso’ (PDB code 1NQE) and ‘in surfato’ (PDB code 1GUF). It is the only known example of structure-based evidence that the lateral pressure difference between lipid and detergent is structurally relevant (a hypothesis by J. Rosenbusch). The orientation of the TonB box of BtuB, which interacts with the TonB protein, relative to the rest of the structure, is different in the ‘in meso’ compared with ‘in surfato’ structures [34].

**Lateral diffusion in the OM of the labelled colicin**

Single-cell/single-molecule detection of lateral diffusion of BtuB and OmpF in the OM of single cells of *E. coli* utilized cells immobilized on cover slips. Receptors were labelled with anti-BtuB and anti-OmpF polyclonal antibody tagged with the fluorescent probe Alexa Fluor® 555, or labelled colicin E3. The lateral diffusion coefficient of BtuB was found to be 5 × 10<sup>−14</sup>−10<sup>−15</sup> m²/s, and three to five times greater in a TonB box mutant [35], indicating a cytoskeleton-like interaction between BtuB and TonB. Motion of OmpF was also detected, but was relatively immobile compared with BtuB.

**Pathway I**

It was inferred from the single-cell diffusion experiments [35] that the delivery of colicin from its primary high-affinity receptor, BtuB, to the OmpF translocator involves mobile BtuB receptors laterally diffusing rapidly on the OM surface. Colicins use the extended coiled-coil R-domain on the mobile BtuB as a ‘fishing pole’ for the OmpF. In this colicin import model, the disordered N-terminal 83 residues of the colicin T-domain functions as the ‘fishing leader,’ delivering the colicin to the OmpF through its initial insertion into the OmpF pore. A special function of the trimeric aspect of OmpF in the colicin import process is not known.

**Pathway II**

Colicin N (PDB code 1A87), whose N-terminal domain is mostly helical, and whose R-domain has little or no coiled-coil conformation, appears to use only one Omp for entry through the OM. It is the porin, OmpF [23,24], which is thought to serve as both receptor and translocator. The N-terminal domain of colicin N is proposed to unfold and bind-insert into the OM through the OmpF central pore, and subsequently to bind the periplasmic TolB protein in the periplasm, believed to function in pulling the colicin through OmpF [36]. Passage of colicin N through the OmpF pore was suggested previously by colicin N occlusion of OmpF channels in planar bilayer experiments [33]. Some specificity is implied in the occlusion as it occurred only from the side opposite (trans) that of OmpF addition to the membrane, and when a negative potential was applied to the side opposite (the cis side) that on which the colicin was added. It has been suggested recently that the pore-forming domain of the colicin traverses the OM through the OmpF–LPS interface in the outer leaflet of the OM [23]. The proof and corroboration of the LPS requirement for colicin N cytotoxicity was obtained from analysis of the Keio collection of the individual genes of the *E. coli* genome [37]. It was thus shown that colicin N interacts with LPS, and requires LPS inner core and the first added glucose, through the concerted action of *galU* and *waaQ* genes [37]. It can be
Figure 1 | Binding of ordered (41–190) and disordered (N-terminal 40 residue segment) peptides of colicin E1 to TolC: secondary structure determined by far-UV CD spectra

Far-UV CD spectra were measured in a quartz cuvette with an optical pathlength of 0.1 mm. Buffer: 20 mM sodium phosphate (pH 7.5) and 0.1 M NaCl. Inset: elution of TolC alone, TolC and colicin E1 peptide ‘41–190’, and peptide alone, via SEC using a Superdex 200 column (10 mm × 300 mm) in 10 mM Tris/HCl (pH 7.5), 0.1 M NaCl and 0.03% DDM (β-dodecyl maltoside). OD280, D280, mAU, milli-absorbance units. Results of SDS/PAGE of peak fractions from Superdex 200 are shown in the upper left corner.

proposed that the interaction of the colicin T-domain with the Tol complex allows the C-domain to unfold and enter the periplasm through the OmpF–LPS interface.

Pathway III: the unique TolC-mediated import pathway of Colicin E1

Genetic analysis established that TolC, previously designated Tol VIII [38], rather than OmpF, is utilized for transmembrane passage of colicin E1. The TolC Omp is a drug-export protein that spans the periplasm with a large (27 Å × 27 Å, backbone) aperture in the OM, where it is anchored by a β-barrel structure [39]. Thus the OM aperture is much wider than that of OmpF, and in contrast with the conclusions reached for the nuclease colicin–OmpF system, it can be inferred that ordered segments of colicin E1 can insert into the OM through TolC (Figure 1, CD spectra). However, the cytoplasmic side of TolC is narrow, a 3.9 Å effective aperture [39,40], and so there would be a major structure problem associated with folded/ordered colicin exit on the cytoplasmic side of TolC [41]. This problem is exacerbated by the fact that, in contrast with the arrangement and utilization of membrane proteins to facilitate drug export, the AcrA/AcrB proteins that function in opening of the iris-like structure of cytoplasmic side of TolC, have been shown through analysis of the Keio collection not to be required for the cytotoxicity of colicin E1 [37].

The N-terminal 40-residue segment of colicin E1 is disordered, as shown by the far-UV CD spectrum (Figure 1, red), but the remainder of the T-domain (residues 41–190) is ordered and helical, 78% α-helix as determined using far-UV CD spectra (Figure 1, green). The entire N-terminal T-domain (190 residues) is 68% helical (Figure 1, blue). The N-terminal 81-residue segment has a helical content of 20% (Figure 1, brown), implying that the unordered structure at the colicin E1 N-terminus extends beyond residue 40, possibly up to residue 65, assuming that the unordered segment is continuous.

To determine the location of the TolC-binding site in the colicin E1 T-domain, the predominantly (78%) α-helical segment 41–190 and predominantly unordered segment 1–81
Figure 2 | Occlusion of TolC channels by colicin E1 N-terminal fragments

(A) Peptide 1–81. (B) Peptide 41–190. Bathing solution: 5 mM Tris/HCl (pH 7.5) and 0.1 M NaCl. TolC was incorporated in planar membrane bilayers by fusion of TolC-containing proteoliposomes. Lipid composition of liposomes and planar bilayer: DOPG/DOPC/DOPE, 2:3:5, molar ratio. A transmembrane potential of 50 mV was applied to the cis side. Channel activity of TolC alone and in the presence of colicin E1 peptides in the cis or trans compartment, is shown in blue, green and red respectively.

More physiologically relevant evidence of TolC interaction with colicin E1 T-domain was obtained through studies on the ion channel properties of purified TolC reconstituted into planar bilayer membranes [42,43]. Peptide 1–81 did not affect TolC channel conductance (Figure 2A), but peptide 41–190 occluded TolC channels when it was added to the cis compartment, the side opposite that on which TolC was added (Figure 2B). Addition to the presence in the trans
Figure 3 | Model for binding-insertion of colicin E1 via TolC
Only the structure of the CTD (C-terminal domain) of colicin E1 has been solved [21], the structure of the remaining domains of the intact colicin shown here are those of colicin E3 [15]. An initial stage in colicin E1 import is shown, in which a mostly ordered, helical, N-terminal domain is inserted into the TolC transperiplasmic channel. The energy transduction function required for import of the colicin E1 channel-forming domain, requiring input of the transmembrane proton electrochemical gradient, \( \Delta \sim \mu_{\text{H}^+} \), generated in the inner membrane, is depicted as a function of the membrane-embedded structure of TolA and those of TolQ and TolR.

compartment was less efficient. Occlusion of TolC channels by helical segment 41–190 implies its insertion into the TolC channel (Figure 3). A structurally defined precedent for this inference is the occlusion of ion channels of the OmpF porin incorporated into planar bilayers channel by the N-terminal T83 peptide of colicin E3 [30]. The inference that this channel occlusion was caused by T83 insertion into the OmpF pore was confirmed by co-crystallization of OmpF and N-terminal 83-residue peptides of colicin E3 [30] and E9 [32].

Effect of TolA truncation on colicin activity
Given the different (TolC) mode of periplasmic passage of colicin E1, it might be expected that its TolA requirement will be different from that of the OmpF-dependent colicins. In fact, deletion (Δ54–287) of most of the transperiplasmic domain of TolA, which is believed to be required for transduction of energy from the inner membrane to the OM, results in total loss of activity of colicins N and A, as expected, but only a small loss for colicin E1 [44]. This extended periplasmic domain of TolA is not necessary for colE1 import, perhaps because part of its function, ‘guidance’ from the OM to the IM, is performed in the TolC periplasmic tunnel. The required energy transduction function is presumably a function of the membrane-spanning domains of TolQR and the membrane-spanning domain of TolA.

Previously established genetic analysis demonstrated that TolC rather than OmpF is utilized for transmembrane passage of colicin E1 [38]. The planar bilayer results shown in the present paper reinforce that inference. However, the ability of this passage to encompass an ordered domain of E1 makes the problem of the entry of the colicin E1 C-domain into the cytoplasmic membrane a major enigma. This problem is compounded by the inference from the Keio library experiments [37] that the AcrAB proteins, which have been proposed to facilitate the opening of the narrow portal proximal to the cytoplasmic membrane for drug export, are not required for colicin import. The problem of the opening of this channel implies a major energy requirement for E1 import, suggesting a requirement for proximity of TolC to the membrane-embedded TolQR, and the membrane-anchored domain of TolA, as depicted in Figure 3. Here, a specific structure prediction is made. Because colicin E1, unlike other colicins, is shielded by its TolC channel from contact in the periplasmic compartment with the Tol energy transduction apparatus [45], and a major segment of periplasmic domain of TolA been shown to be unnecessary for colicin E1 activity [44], it is hypothesized that transduction of the proton electrochemical potential, \( \Delta_{\sim \mu_{\text{H}^+}} \), required for the cellular import of colicin E1 occurs through interaction with the membrane-embedded TolQR and the membrane sector of TolA, which are predicted to be organized in a supercomplex (Figure 3).

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References