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Review

Colicin crystal structures: pathways and mechanisms for colicin insertion into membranes

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Abstract

The X-ray structures of the channel-forming colicins Ia and N, and endoribonucleolytic colicin E3, as well as of the channel domains of colicins A and E1, and spectroscopic and calorimetric data for intact colicin E1, are discussed in the context of the mechanisms and pathways by which colicins are imported into cells. The extensive helical coiled-coil in the R domain and internal hydrophobic hairpin in the C domain are important features relevant to colicin import and channel formation. The concept of outer membrane translocation mediated by two receptors, one mainly used for initial binding and second for translocation, such as BtuB and TolC, respectively, is discussed. Helix elongation and conformational flexibility are prerequisites for import of soluble toxin-like proteins into membranes. Helix elongation contradicts suggestions that the colicin import involves a molten globule intermediate. The nature of the open-channel structure is discussed. Published by Elsevier Science B.V.

Keywords: Coiled-coil; Colicin; Ion channel; Molten globule; Outer membrane; *E. coli*; Protein import; Receptor

1. X-ray structures of colicins and their C-terminal domains; what do they tell us about the mechanism of colicin insertion into and through the cell envelope?

1.1. Intact colicins (Fig. 1A–C)

Two structures of an intact or almost intact colicin have been solved by X-ray diffraction of three-dimensional crystals: (i) The structure of residues 23–39 and 83–624 of the 626 residue pore-forming colicin Ia (“B-type,” translocated by the TonB system) has been resolved to 3.0-Å resolution (Fig. 1A [1]). (ii) Residues 84–551 of the 551-residue non-pore forming “”A-type” (translocated by the TolA system) endoribonuclease colicin E3 have been solved to 3.0 Å; approximately half of the N-terminal translocation domain (T, in green, Fig. 1) was not resolved (Fig. 1B [2]). The region of colicin E3 drawn in yellow in Fig. 1B corresponds to its immunity protein [2]. (iii) In addition, 292 residues, 93–385, of a 321-residue chymotryptic fragment of the smaller 387-residue pore-forming colicin N, provide a 3.1-Å structure that contains the globular channel domain (C, in blue, Fig. 1C), but is missing the T domain and a substantial part of the N-terminal region of the R domain (in red, Fig. 1 [3]).

Colicin Ia forms an elongate 210-Å structure with spatially well-separated domains (C and T, Fig. 1A) that are responsible for the functions of channel formation (C) in the cytoplasmic membrane and translocation (T) across the periplasmic space. The prominent elongate nature of the structure is a consequence of an extended 160-Å long coiled-coil structure consisting of residues 176–282 and 359–467 (red, Fig. 1A). In colicin E3, a somewhat shorter but still quite extended (100 Å) anti-parallel “Alacoil” helical coiled-coil structure, in which every seventh residue is an alanine in the core of the coiled-coil, was subsequently described in the structure of colicin E3 [2]. By analogy with colicin Ia, and the R-domain coiled-coil structure inferred from calorimetry and circular dichroism (CD) for colicin E1 [4], the coiled-coil spanning residues 320–440 of colicin E3 was proposed to contain the R-domain function [2]. The 135-residue colicin E3 peptide, Thr^{313}–Asn^{447}, was sub-

Abbreviations: CD, circular dichroism; DSC, differential scanning calorimetry; FRET, fluorescence resonance energy transfer; FTIR, Fourier transform infrared spectroscopy; Omp, outer membrane protein; P178, P190, 178 and 190 residue C-terminal channel forming domain of colicin E1

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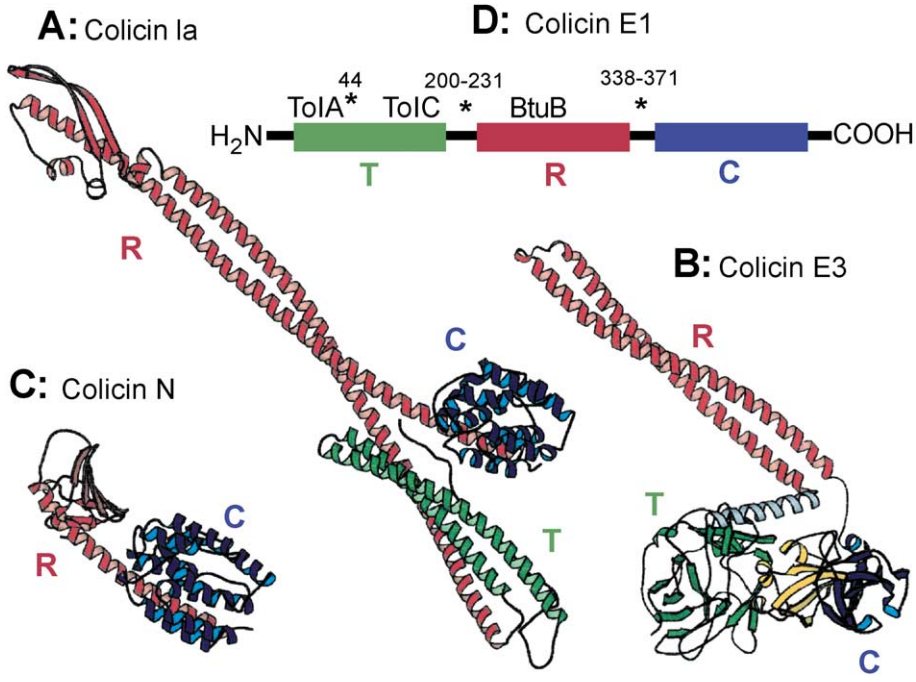

Fig. 1. Ribbon diagrams describing the atomic structures of (A) colicin Ia [1], (B) colicin E3 [2], and (C) colicin N [3], derived from X-ray diffraction analysis of 3-D crystals. The regions corresponding to R (red), T (green), and C (blue) domains are shown. Residues 1–22 and 40–82 in colicin Ia, 1–66 in colicin N, and 1–83 in colicin E3, respectively, were not resolved; figure generated by MOLSCRIPT [67]. (D) Functional domain organization of the 522-residue colicin E1. The colicin E1 R domain was predicted from proteolytic [8] and cyanogen bromide [68] cleavage, and was verified by combined CD and DSC analysis [4]. N termini of proteolytic fragments of E1 P342 and P178 are indicated by asterisks.

sequently found to have a high affinity for the isolated BtuB receptor and to retain its coiled-coil nature [5].

1.2. The domain concept

Separable or distinguishable domains in the colicins can be defined by two general criteria: (a) function and (b) structure (e. g., Fig. 1D). The definition of functional domains, mostly utilizing a combination of genetic, microbiological, and biochemical analysis [6], historically preceded those defined by structure.

Even though relatively compact domains defined by X-ray structure (e.g., green, red, and blue domains, Fig. 1) can often be associated with functional domains, this association is not always unambiguous. Thus, (a) in colicin E3, it is not clear a priori, whether the short helix (residues 293–313, in gray, Fig. 1B) that is oriented at a sharp angle to the extended coiled-coil (red) should be associated with the T- and/or R domains that it connects; (b) in colicin Ia, the N-terminal 50-residue (176–225) segment of the long helix spanning residues 176–282 (red, Fig. 1A) was originally associated with the two anti-parallel α-helices of the T domain [1]. However, because of the continuity of this long helix, and the apparently limited contact of its N-terminal 50 residues with the two anti-parallel helices of the T domain (green, Fig. 1), we have designated the entire 176–282 helix as part of the receptor-binding (R) domain along with the rest of the extended coiled-coil (red, Fig. 1A). One caveat to this designation is that the degree of interaction of the N terminus of the 176–282 helix with the T domain is somewhat uncertain because only 17 of the first 82 residues in the T domain have been resolved.

The colicin structural domains can be distinguished by: (i) selective proteolysis and (ii) differential scanning calorimetry (DSC). The relatively high proteolytic sensitivity of the T domain has allowed isolation of C and R-C domains (e.g., Ref. [7,8]). Intact colicin E1 and its T, R, and C domains melt cooperatively as separate units with defined heat capacities and distinct melting (T_m) temperatures (Fig. 2, [4]), because they are separately packed by van der Waals, H-bond, and electrostatic interactions. The structural and functional definitions of colicin domains generally overlap, but the T or R domains may contain multiple functional regions or sites, as shown in the 34-residue N-terminal TolA and 140-residue TolC [9] subdomains in the T domain of colicin E1 (Fig. 1D).

1.3. R domain

Considering the similarity of the length of the coiled-coil with the approximate width of the periplasmic space, it was proposed that the function of the major part of the 160-Å

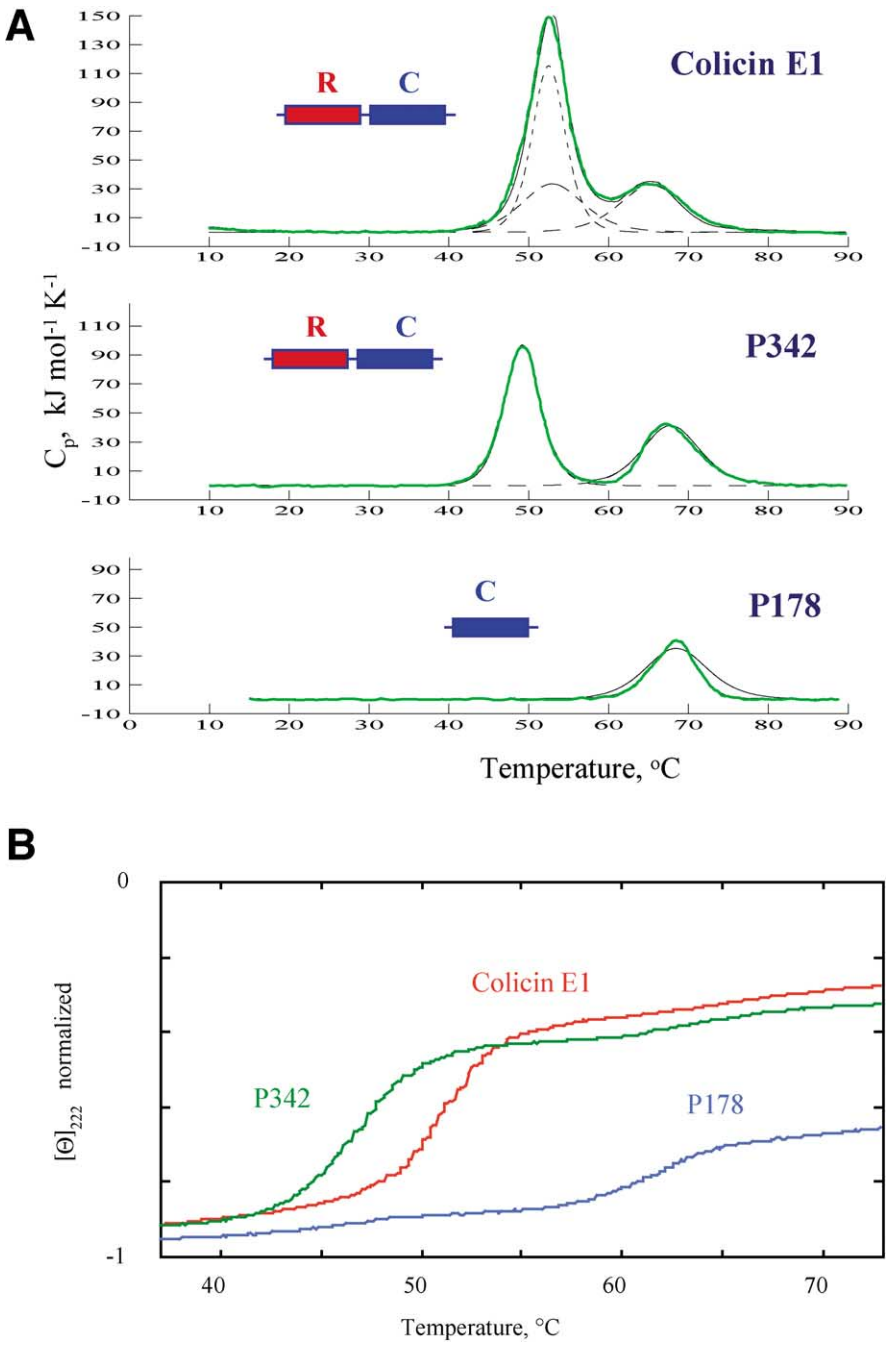

Fig. 2. Differential scanning calorimetry and circular dichroism melting curves of colicin E1 and its three structural domains. (A) Deconvolution analysis of the excess heat capacity function of intact colicin E1. Broken lines represent the fitting of the experimental data (green) to three transitions, with the sum of individual transitions shown as black solid lines. (B) Thermal scanning of CD signals of intact E1, P342 and P178 at 222 nm, 23 °C. Modified from Ref. [4].

coiled-coil of colicin Ia (residues 176–282 and 359–467) would be to bridge the periplasmic space as part of the mechanism of translocation across it [1]. In this proposal, only a 104-residue region at the tip of the coiled-coil, consisting of an amphipathic two-stranded β-hairpin folded around the tip-terminal helix of the coiled-coil, was proposed to be involved in binding to the colicin Ia *Cir* receptor. The R domain of colicin N was proposed to consist of a 65-residue, 97–161, six-stranded β-strand wrapped around the C-terminal end of a 45-residue extended helix (65 Å) that was proposed to be part of the pore-forming domain [3].

Regarding colicin Ia, comparison of its structure with genetic and function analysis of the domain structure of the pore-forming colicins implied that the whole coiled-coil might be a good fit to the receptor binding R domain. Subsequent studies of the minimum receptor binding domain in the A-type colicins E9 and E3 that inhibit

intracellular protein synthesis have indicated that a large part of the coiled-coil, but perhaps only its distal half, might be necessary for recognition and effective binding to the receptor (vide infra, Sections 3.3–3.4). The coiled-coil nature of the receptor domain in the structure of intact colicins Ia and E3 implies that this is a necessary structure motif for interaction with the receptor that initially sequesters the colicin (vide infra). In the case of colicin N, only one extended helix can be seen (Fig. 1C), which is only about 65 Å in length. The second helix of the coiled-coil may be missing in the colicin N structure because of the use of the chymotrypsin fragment. The markedly smaller length of the extended helix or coiled–coil of colicin N is suggestive of a mechanism that is different from those used by colicins Ia, E3 and E9 for receptor binding and translocation across the outer membrane and periplasmic space.

1.4. Coiled-coil nature of R domain of colicin E1

The presence of an extended coiled-coil structure as the dominant feature of the colicin E1 R domain was inferred from DSC and CD analysis of the stability and interactions of the colicin E1 R, T, and C domains [4]. The cooperative thermal melting of highly helical R domain of the colicin E1 implies that it is similar in structure to the R domain of colicin E3 defined by X-ray structure analysis [2]. The highest and lowest enthalpies (ΔH) of denaturation of colicin E1 were associated, respectively, with the central R- and N-terminal T domain. The large ΔH_R of the R domain is ascribed to extensive hydrogen bond and van der Waals interactions associated with the coiled-coil structure. The steep temperature dependence of its stabilization energy in the physiological range, its interactions with the T domain that mutually stabilize both domains, and its destabilizing interaction with the C domain, imply that the R domain has a dominant role in determining the conformation of the whole protein and individual domains. Its very high (>90%) α-helical content, and highly cooperative thermal melting detected by far-UV CD in the same temperature range as the R-domain denaturation measured by DSC, imply that the R-domain coiled-coil is a major determinant of the structure of the colicin. The high sensitivity of the R domain to environmental conditions and the linkage of the coiled-coil helices to the T and C domains indicate that the R domain could control conformational changes of the T and C domains [4]. Unfolding of the R-domain coiled-coil could trigger a sequence of events involving sequential interactions with the BtuB and TolC receptors that result in import of the C domain across the outer membrane to the cytoplasmic membrane (vide infra, Section 3).

1.5. T domain

The N-terminal T domain of colicin Ia contains the "TonB box" (E23–V27) necessary for translocation across the periplasm as part of an antiparallel helix bundle. The secondary structure motif contrasts with the β-sheet "jellyroll" that forms most of the colicin E3 T-domain. The colicin E3 T-domain contains in its N-terminal region a DGSGW (D35–W39) Tol B—"box" needed for translocation, and is glycine-rich (34 of 79 residues), accounting for the disorder in this region. A possible reason for the difference in the T-domain structures of colicins Ia and E3 is that the immunity protein is tightly bound to the T domain in the latter. The immunity protein of the channel forming colicins is not attached to the colicin and resides in the cytoplasmic membrane of *Col* or *Imm* cells [10, 11]. The colicin E1 T-domain was found to be marginally stable, and is significantly stabilized by interactions with R and C domains. Its enthalpy of melting, $\Delta H_T = 320$ kJ/mol is on the lower end of the range for globular proteins, implying its structure is flexible due to a poor network of intradomain van der Waals and H-bond interactions [4]. This is in agreement with the high sensitivity to proteolytic degradation of the colicin E1 T-domain [8] and with the lack of resolution of the N-terminal 22 residues of colicin Ia [1] and 83 residues of colicin E3 [2] in the electron density derived from X-ray diffraction.

1.6. Structures of C-terminal channel domains

The soluble active (pore-forming) 20,000 molecular weight domain of colicins A [12] and E1 [13] along with the structures of these domains in the intact colicins Ia and N have the characteristic signature of a 10 helix globule. The average length of these helices is 13 residues, much shorter than required (ca. 20 residues) to span the membrane bilayer.

The two longest helices, VIII–IX, in the channel domain form a hydrophobic helical hairpin in the apolar core of the channel domains, a feature that is also found in the structures of the membrane-active domain of diphtheria toxin [14] and of the apoptotic protein, BCl-X_L (Fig. 3 [15]). This suggests that the function of this hydrophobic hairpin structure motif in membrane-active proteins is to provide a membrane anchor after the initial interaction of the protein with the membrane surface (vide infra). Thus, the mechanism of interaction of the pore-forming colicin channel domains may be a paradigm for membrane-active apoptotic proteins.

1.7. Helix dipoles can be involved in stabilization of the colicin E1 channel domain

CD and DSC analysis have shown the 178-residue channel polypeptide, P178, to be more stable at low than at high ionic strength (10 vs. 300 mM), implying a prevalence of attractive over repulsive interactions even at pH 2.5. It is hard to explain such an effect by electrostatic attraction between basic (27 residues in P178) and acidic (22 in P178)

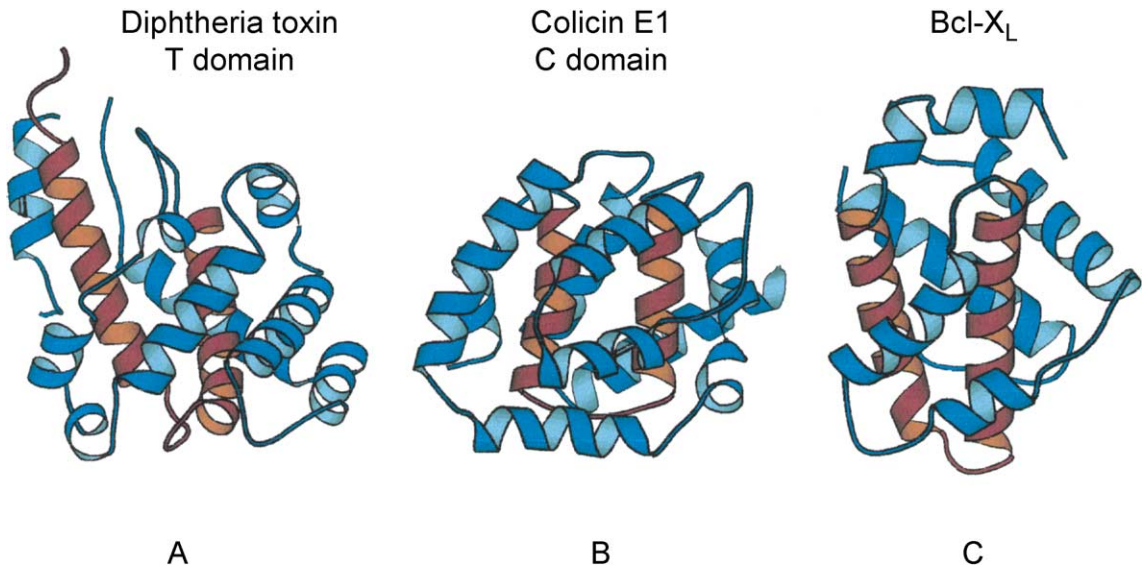

Fig. 3. Ribbon diagram of (A) T-domain of diphtheria toxin [14], (B) channel domain of colicin E1 [12], and (C) BCl-X_L [15]. Hydrophobic hairpin shown in brown. Shell of hydrophilic helices is in blue.

side chains, because at least 90% of the carboxyl groups are protonated at pH 2.5. There are no anomalously titrated carboxyl groups in the colicin E1 channel domain in solution (S. Venyaminov and S. Zakharov, unpublished data). It is inferred that attraction between basic side chains and the negative carboxyl ends of helix dipoles provides the stabilization.

Beyond the implied role of the buried helical hydrophobic hairpin as a potential membrane anchor, the structure of the soluble channel domain provides few clues about the mechanism of colicin insertion into the membrane. Rather, the structure implies that the soluble structure must undergo large conformational changes in order to accomplish the insertion. The mechanism of membrane insertion of the C-terminal channel-forming domain, and the properties of the inserted channel, have been studied with defined synthetic lipid membranes using liposomes and planar bilayer membranes.

2. Studies with synthetic membranes; what do they tell us about mechanism of insertion into the cytoplasmic membrane?

2.1. Kinetics of initial steps in membrane interaction

The initial events associated with the enormous conformational changes undergone by the insertion of colicin into membranes have been studied through the interaction of the 178-residue C-terminal channel domain of colicin E1, P178, with liposome membranes of defined lipid composition. The interaction is triggered in vitro by utilizing an acidic pH ~ 4 to (a) create attractive electrostatic interactions between cationic P178 (effective binding charge, +7) and liposome membranes with a near-physiological (30–40 mol%) anionic lipid content [16,17], and (b) to facilitate unfolding of the protein at the membrane surface [18]. The first-order rate constants for the initial events of membrane interaction

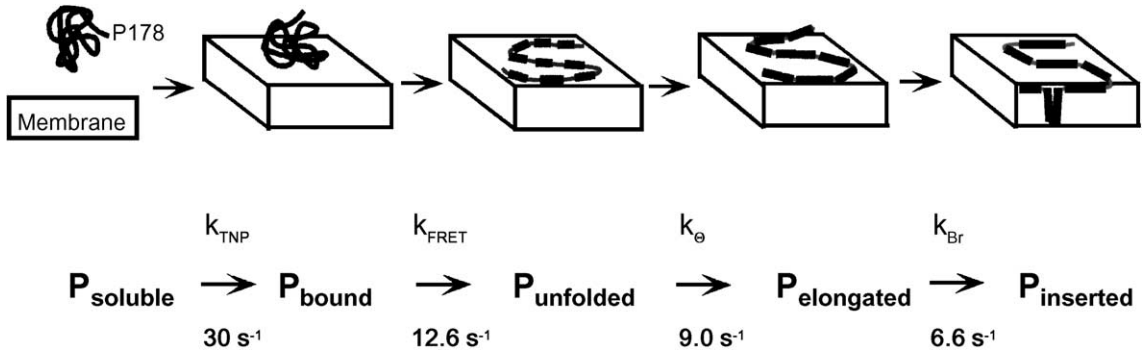

Fig. 4. Pathway of the distinguishable structure transitions of P178 in the course of binding to the membrane surface and insertion into the bilayer. Rate constants for defined steps are for pH 4.0, I = 0.1 M and 25 °C (modified from Ref. [20]).

of the colicin E1 channel polypeptide, P178, which are defined by stopped flow measurements of fluorescence quenching, fluorescence resonance energy transfer (FRET), and CD are: (i) membrane binding ($k = 30 \pm 5\ s^{-1}$), (ii) unfolding ($13 \pm 0.5\ s^{-1}$; obtained by FRET with Trp^{424}; rate constants for other Trp in Ref. [19]), (iii) helix elongation ($k = 9.0 \pm 1.0\ s^{-1}$), and (iv) insertion ($k = 6.6 \pm 0.5\ s^{-1}$) [20]. The net result is an unfolding of the globular channel domain into an extended flexible helical two dimensional network in the 10–15-Å-thick interfacial layer (Fig. 4). In addition, it is energetically favorable for helical units to organize on the membrane surface as paired anti-parallel helical dipoles in individual helical hairpins.

2.2. Prerequisites for protein import

2.2.1. Helix elongation

With an average length of 13 residues, the α-helices of the colicin E1 channel domain are much too short to span the membrane bilayer. One of the structure changes that must occur upon membrane binding is an elongation of the helices that will ultimately span the membrane. In fact, the α-helices of P178 elongate upon membrane binding by approximately 30% from 13 to 17 residues (Fig. 5 [21]), as determined by CD and infrared spectroscopy, assuming that membrane-bound P178 has the same number of helical segments as in the soluble state. After the activation barrier imposed by structural restraints is removed by the unfolding, helix formation is favored thermodynamically in the intermediate polarity environment of the membrane interfacial layer [22–24]. Helix elongation of a membrane-active protein upon membrane binding is predicted to be a general property of pore-forming toxins that must ultimately form a trans-membrane structure in which at least four helices span the membrane as α-helices with a length of 18–20 residues. Another consequence of the helix extension is the increase of the average dipole moment associated with the helices.

2.2.2. Conformational flexibility

The models for P178 in the initial surface-bound state and the final membrane-inserted open channel state (Fig. 6A–C [21]) imply a large-scale rearrangement within the plane of the interfacial layer and in the direction orthogonal to it. It is intuitive that conformational (motional) flexibility of the membrane-bound channel domain and significant freedom of motion in the membrane interfacial layer and bilayer are prerequisites for the cooperative motion required for protein insertion and voltage-gated channel activation from the initial surface-bound state. Conformational freedom is implied by:

(a) Reversible thermal melting and change of inter-residue distances detected by FRET of membrane-bound P178 [21]. The ability of the protein to undergo large internal distance changes as a function of temperature without denaturation is presumably a consequence of its restricted degrees of entropic freedom in two-dimensional space.

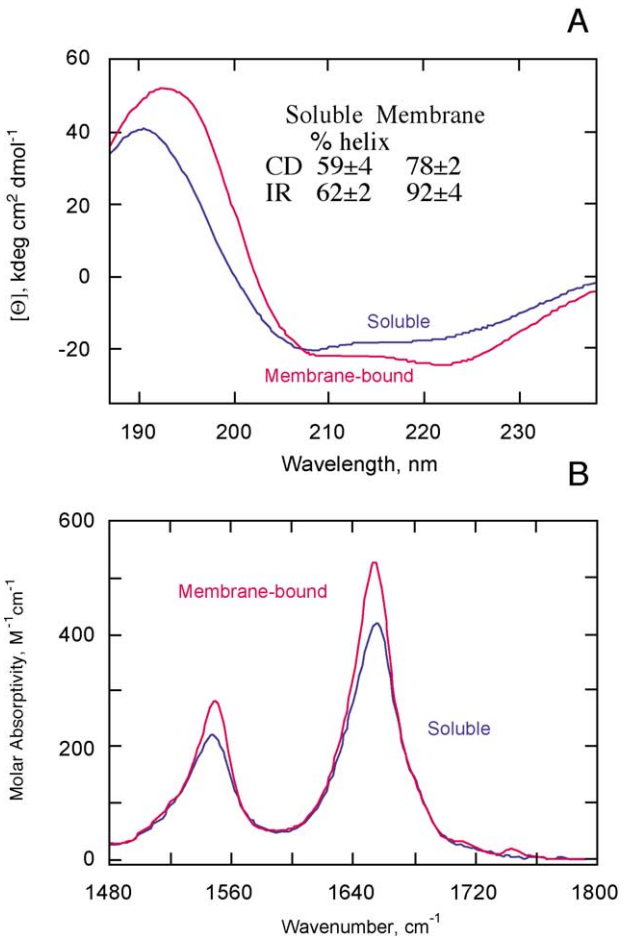

Fig. 5. (A) Far-UV circular dichroism and (B) FTIR spectra of soluble (blue) and membrane-bound (red) colicin E1 channel domain. Liposomes (0.1 μm) were prepared by extrusion of DOPG/DOPC, 2:3 mol/mol. The data on α-helical content are shown in the inset. The FTIR spectra were corrected for absorbance of side chains (modified from Ref. [21]).

(b) Rapid and extensive H/D exchange of P190 bound to liposomes [25].

(c) The reversible thermally induced increase of inter-residue distances is prevented at values of the membrane surface potential, ψ_o, more negative than -60 mV, or anionic lipid concentrations >30 mol% at 0.1 M ionic strength, as is the ability of P178 to insert into the membrane and form channels. Using different anionic lipid concentrations and ionic strengths, channel formation and protein insertion are remarkably precisely tuned at $\psi_o \approx -60$ mV (Fig. 7; [26]). It was concluded that the motional freedom of the surface bound P178 that is necessary for insertion and channel formation is constrained by electrostatic interactions of the positively charged basic groups of the cationic polypeptide with phosphates of the lipid headgroups (Fig. 8A).

(d) Motional flexibility of the membrane-bound channel domain of colicin Ia was also demonstrated by solid state NMR using liposomes with 25 mol% anionic lipid content [27]. A significant increase in motional amplitudes for both the backbone and side chains was shown on a pico- to

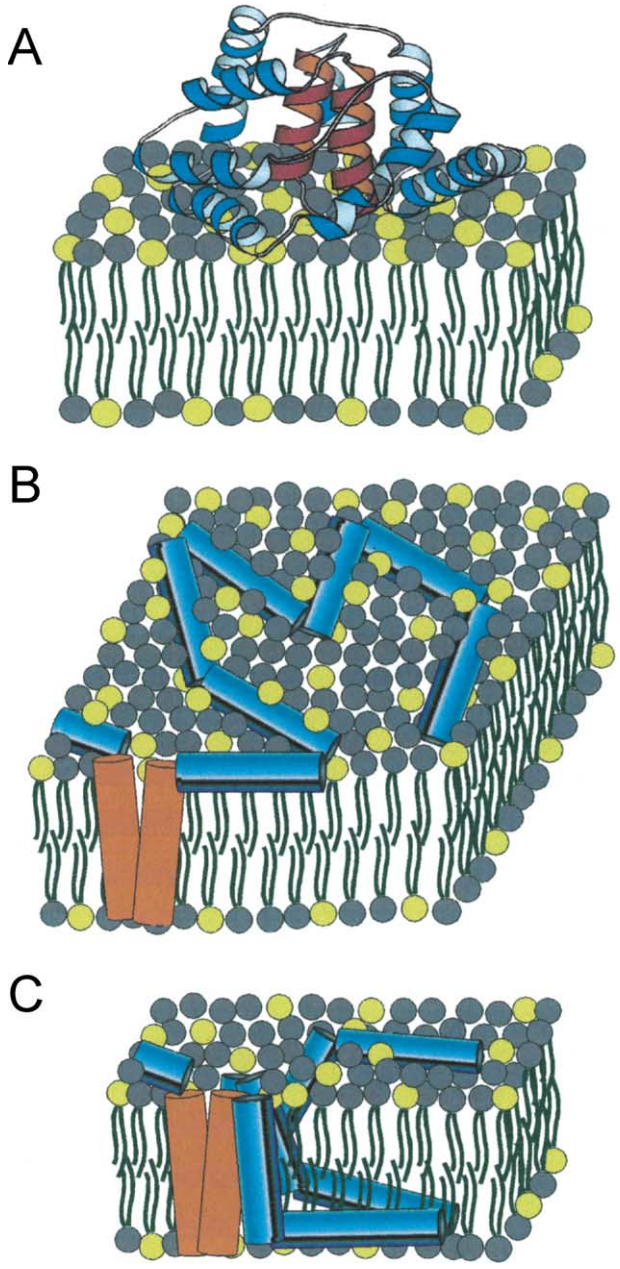

Fig. 6. Sequence of events in membrane import of the colicin channel domain. (A) Surface-bound globular non-inserted P178 [13], which preserves most of the interhelix contacts of the soluble state. This could be the intermediate state for a surface potential, $|\psi_o| \ll 60$ mV as a result of incomplete unfolding of surface-bound P178. (B) An extended two-dimensional helical array in the membrane interfacial layer as a precursor to the open channel state for $\psi_o \approx -60$ mV; this helical array is less flexible and more compact at $|\psi_o| \gg 60$ mV. (C) Depiction of the imported open-channel state; comparison with (B) shows requirement for movement of surface-bound helices in the transition from closed to open channel states. Hydrophobic helices VIII and IX are drawn in brown, and hydrophilic and partially amphipathic helices are in blue.

microsecond time scale. Motions on the microsecond time scale could include independent (uncorrelated) small-scale reorientations of helices in bilayer, rocking motions in and out of the plane of the membrane bilayer. The 12–16° angular excursion of the colicin Ia channel domain backbone, detected in this study, differs from the behavior of bacteriorhodopsin and the phage fd coat protein. The amplitudes of motion of the side chains upon membrane binding increase even more than for the backbone. A high mobility of lysine side chains of the membrane-bound colicin E1 channel domain was previously shown by solid-state NMR of the colicin E1 P190 [28].

2.3. No detectable molten globule intermediate

The intriguing suggestion that the low pH activity of the colicin A channel domain might occur through an unfolded molten globule intermediate in which the α-helix content is preserved [29] is not supported by the data for colicin E1. In principle, the exposure of the hydrophobic core in molten globule would imply that this intermediate state would have a larger affinity than the native water-soluble state for the lower polarity membrane surface [30]. However, for the soluble channel domain of colicin E1, P190, the active low pH state is not accompanied by a loss of tertiary structure as monitored by near-UV CD [31]. Furthermore, loss of tertiary structure and conservation of secondary structure are necessary, but not sufficient, determinants of the molten globule state. In addition, the molten globule preserves the compact hydrophobic core [30]. The data show that the only unfolded intermediate that can be detected in the interaction with membranes is characterized by (i) a substantial increase (ca. 30%) in helical content, as discussed above, which is required to convert the soluble P178 to an integral trans-membrane channel; (ii) a threefold increase in the membrane-subtended protein cross section upon membrane binding as measured by surface plasmon resonance [21]; (iii) Br- (unpublished data) and Doxyl- [32] quenching of fluorescence of P190 shows that tryptophans of hydrophilic helices are accessible to contact with quenchers, implying that helices are deep in the interfacial layer parallel or oblique to the membrane plane, rather than forming three-dimensional compact domain on the membrane surface. It is possible to consider, however, that the unfolding of the colicin that follows membrane binding ($30\ s^{-1} < t_{1/2} < 10\ s^{-1}$) under the conditions described in Section 2.1, occurs through a molten globule intermediate. Such an intermediate would not be detected because of the difficulty of measuring time-resolved near-UV CD changes on a millisecond time scale.

2.4. The open channel state

2.4.1. Protein import is integral to voltage gating

Voltage-gated opening of the ion channel of the pore-forming colicins is known to be mechanistically different from that of the classical integral membrane channels. Whereas gating of the latter involves small conformational changes of the trans-membrane segments [33], gating of colicin channels involves import of a large part of the protein from the membrane surface into and across the

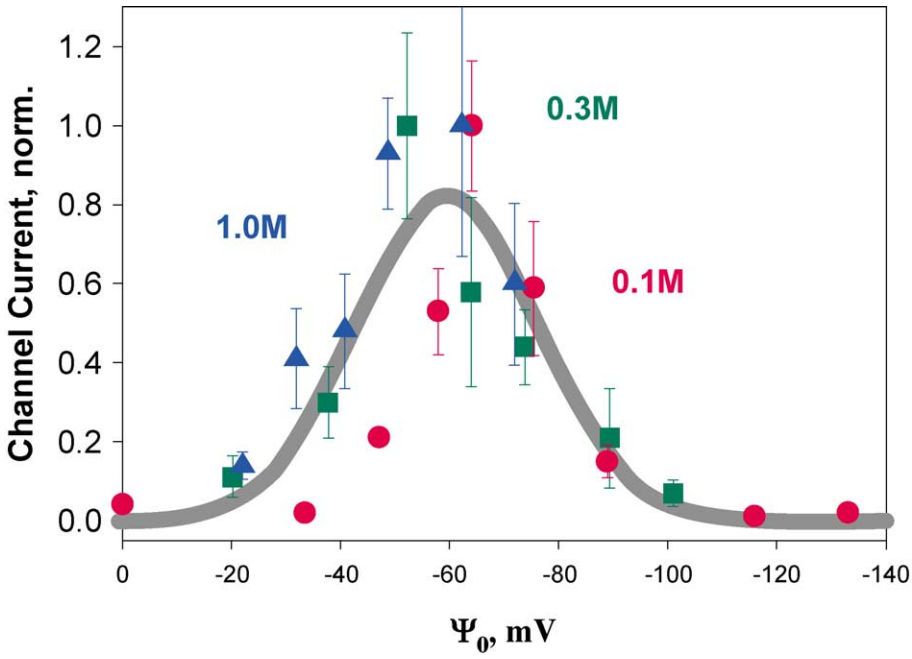

Fig. 7. Macroscopic steady-state current in planar bilayers as a function of surface potential calculated for using Gouy–Chapman theory [69]. Data were normalized relative to the amplitude of the maximum current for different ionic strengths, i.e. at 25, 30, and 70 mol% DOPG for 0.1 (● and red), 0.3 (■ and green), and 1.0 M (▲ and blue) KCl, respectively.

hydrophobic core of the bilayer [18,34]. As discussed above, this large-scale import is the underlying cause of the requirement for conformational flexibility of the surface-bound closed channel intermediate. Much information on the qualitative structural nature of the inserted channel has come from a series of experiments using *cis*- and *trans*-side streptavidin trapping of biotin-labeled single cysteine mutants of the 177 residue colicin Ia C-terminal channel domain studied in planar lipid bilayers [35]: (i) Using the colicin Ia C-terminal domain that was biotin-labeled at the apex (residue 594) of the helix VIII–IX hydrophobic hairpin, this system has provided the only definite proof for a voltage-independent *trans*-side penetration of the VIII–IX hairpin [36]. It has not been possible to detect this *trans*-side penetration using liposomes (Lindeberg, Zakharov, and Cramer, unpublished data). Thus, *trans*-side penetration of the hydrophobic hairpin may only involve a small fraction of the colicin population at any one time, although all molecules assume this state and are eventually trapped.

The voltage-gating event consists of the translocation to the *trans*-side streptavidin-accessible domain, residues Leu^{474}–Tyr^{541}, a very long 68-residue peptide segment

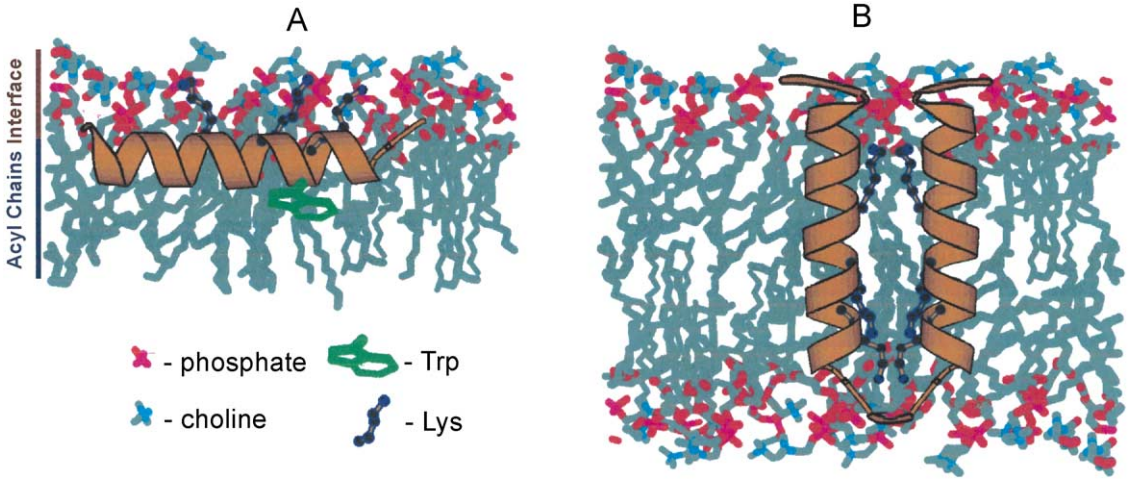

Fig. 8. Representative amphiphatic helical segments of channel-forming domain (A) in interfacial layer, and (B) inserted into membrane bilayer by a membrane potential (voltage-gating) as an anti-parallel helical hairpin in the open-channel state. Trp shown inserted into membrane (A), to illustrate results of experiments on quenching of colicin E1 Trp fluorescence [32]. Lysine side chains are shown to snorkel to the surface [70].

consisting of helices II–V in the soluble Ia channel peptide. The translocated segment is bounded by up- and downstream trans-membrane helices, ca. residues Asn^{454}–Leu^{474} and Tyr^{541}–Arg^{560}, upstream from the hydrophobic hairpin anchor, $Ileu^{574}$–$Ileu^{612}$. Thus, including the bordering trans-membrane helices, the biotin–streptavidin experiments show that approximately 105 residues of the colicin Ia channel domain can be imported across or translocated into the membrane by the voltage-gated channel opening. In addition, an additional nine-residue heme-agglutinin peptide or highly charged FLAG (DYKDDDDK) can be translocated [34]. A partly corroborative result for the colicin E1 channel domain was obtained using liposomes by differential labeling in the presence and absence of a potassium diffusion potential [18]. Residues 420–460, corresponding to colicin Ia residues 524–564, were inferred to reside in the membrane inserted segment, and believed to include two trans-membrane segments of Ia.

The question has been raised as to whether the translocation of the 68-residue segment of colicin Ia occurred simultaneously or, because most trapping experiments were done with single Cys mutants, the translocated loop and the upstream trans-membrane helix might be variable, "the spaghetti model" [37]. This alternate view was tested by looking for *cis*-side streptavidin-trapping of biotin attached at any one of many Cys residues inserted by site-directed mutagenesis in the 68 residue translocated segment [18,35]. If the actual translocated segment was small, variable, and time-dependent, a Cys residue in this region should be found in the *cis*-side aqueous phase where it could be trapped. No such Cys residues were found. However, if the Cys residue flipped back only as far as the *cis*-side interfacial layer of the membrane, then it would probably not be accessible to streptavidin. The "spaghetti model" has been rendered unlikely by recent data showing that a 134-residue hydrophilic polypeptide inserted into colicin Ia can be transported to the *trans*-side of the membrane bilayer [38].

2.4.2. Problem of translocation of charged residues; role of helical hairpins and dipoles

Insertion of the colicin surface-bound helices into and across the membrane bilayer as part of the voltage-gating process confronts the large energy barrier (the Born energy, 40 kcal/mol for the singly charged terminal ionic group of lysine with a 2.0-Å radius [39]) associated with translocation of charged residues across the hydrophobic membrane bilayer. As noted above, it is energetically favorable for helical units on the membrane surface to be organized on the membrane surface as paired anti-parallel helical dipoles in individual helical hairpins. Voltage-gated translocation of helices II–VI of the colicin channel domain as helical hairpin units (prototype inserted hairpin shown in Fig. 8B) could reduce the energy barrier because of charge pairing and neutralization of the some of the amino acid charges. The 68-residue translocated segment of helices II–V of colicin Ia contains 22 charged residues, 15 basic and 7 acidic.

2.4.3. Nature of the pore structure

The structure of the open channel is enigmatic, as discussed more completely in a recent review [40]. A fundamental problem, which ultimately leads to a structurally difficult view of the channel, is that the channel from the intact colicin or the pore-forming domain is monomeric in planar bilayers. This is based on titration of ionic solute efflux in liposomes [41] and of channel current in bilayers [42]. For the intact colicin, the monomeric open channel is proposed to consist of four trans-membrane helices, two of which are contained in the hydrophobic anchor, and two that are hydrophilic framing the large translocated domain [40]. It is difficult to understand how these four helices can form a defined channel. Even more difficult to understand is the inference that the upstream fourth trans-membrane helix would be free on the *trans*-side for the channel domain, and that the channel might then consist of only three trans-membrane helices [34]. In spite of the historical emphasis on single molecule action, it is possible that a significant fraction of the active colicin channels in the planar bilayer, particularly after long incubation times, is actually dimeric or oligomeric.

2.4.4. Caveats to in vitro experiments with synthetic lipid membranes

It is obvious that in the attempt to utilize a pure and spectroscopically tractable system to study protein–membrane interactions, it is quite possible for artifacts to be introduced. A general caveat in a study of protein insertion into artificial membranes is the absence of the membrane proteins that certainly direct the insertion under physiological conditions. On the other hand, the lipid membrane environment is known to define or influence many properties of integral membrane proteins: (i) the thickness of the bilayer dictates the length of trans-membrane α-helical or β-sheet segments; (ii) the polarity or dielectric constant of the membrane and interfacial layer is a determinant of the trans-membrane distribution of hydrophobic and hydrophilic residues and, together with steric factors, that of tryptophan [43]; (iii) the existence of the negative membrane surface potential is the simplest explanation of the ubiquitous *cis*-positive distribution of positively charged residues [39].

Another possible in vitro caveat in studies of the interaction of artificial membranes with pore-forming colicins is the use of acidic pH to initiate the interaction. The use of acidic pH in studies of the membrane interaction of toxin, fusion, and colicin proteins and peptides in vitro is a conceptually well-established method for initiating the interaction of these membrane-active proteins with anionic lipid membranes. The electrostatic interaction between the anionic membrane and the cationic polypeptide generated at low pH replaces to some extent the specific protein interactions in vivo that initiate the interactions. There is no question about the physiological relevance of this procedure for toxin and fusion proteins such as diphtheria that pass through the acidic endosomal compartment. In planar mem-

brane experiments, the behavior of the colicin channel domain is qualitatively similar to the T domain of diphtheria toxin. In experiments with intact colicin, one other consequence of the use of acidic pH is that unfolding is facilitated [44], apparently because the interaction of the C domain with R and T domains is destabilized [4].

2.4.5. Membrane thinning

The binding and insertion into the membrane interfacial layer of small membrane-active peptides, such as the 23-residue peptide, magainin-2, causes thinning of synthetic lipid bilayers, measured by lamellar X-ray diffraction, which can facilitate spontaneous peptide insertion [45]. Bilayer thinning is 1 Å at a peptide/lipid molar ratio of 1:100 and 2 Å at 1:60. This effect could be relevant in liposome experiments with colicin channel peptide. On one hand, the channel peptide/lipid ratio is smaller, typically 1:500 [21]; however, the amino acid/lipid molar ratio is probably similar to that for magainin. Protein-induced membrane thinning is probably not a factor in planar bilayer experiments where protein/lipid ratios are small.

2.4.6. Mechanism of action of immunity protein; implications for formation and structure of open channel state in vivo

Colicin-producing cells protect themselves by coordinate but not stoichiometric production of an immunity protein that inhibits very specifically the cognate colicin to block its lethal effect. The immunity proteins for the channel-forming colicins, small hydrophobic proteins consisting of three (colicin E1) or four (colicin A) trans-membrane helices, are localized in the cytoplasmic membrane [10,46] and interact with the C-terminal channel-forming domain [10,47,48]. The use of hybrid colicins to identify regions of immunity [47,48], and co-immunoprecipitation of the helical hairpin with the colicin A immunity protein [11], show that helix VI, the helix VII–VIII interface, and the helix VIII–IX hairpin of the C-terminal domain are crucial for channel formation. These are exactly the same regions of the channel domain that have been shown to be the transmembrane segments mapped by differential hydrophobic labeling in liposomes [18] and biotin–streptavidin trapping in planar bilayers [34,35]. A model for the mechanism of interaction of the hydrophobic helices of the colicin E1 immunity protein with the colicin channel domain has been presented elsewhere (Fig. 8 in Ref. [49]).

3. Colicin import across the outer membrane and periplasm

Receptor-mediated protein import; the two receptor concept; function of T domain in translocation across outer membrane. The existence of two well-defined outer membrane proteins, BtuB and TolC, for cellular import of colicin E1 provides a framework for considering its receptor-mediated uptake mechanism. In addition to the R domain that contains the sequence information for binding to the BtuB receptor, the colicin E1 T-domain also contains a binding site for the unique outer membrane TolC protein (Fig. 1D). This suggests a two-receptor concept for uptake of this particular colicin, somewhat analogous to the two receptor concept for cellular uptake of HIV [50] and the possibilities for dual or multiple receptor function in signal transduction pathways [51]. For HIV, the gp120 envelope glycoprotein can interact with the CD4 receptor on the surface of target cells such as T lymphocytes. The binding to CD4 can induce conformational changes in gp120 that lead to exposure, recognition, or formation of a chemokine co-receptor.

3.1. Import of colicin E1

The data suggest a two-receptor model for colicin E1. The vitamin B12 receptor, BtuB, can function in initial recognition and binding, and TolC as the second receptor in channel-mediated protein uptake (Figs. 9). The 140-residue TolC binding domain is located in the N-terminal third of the colicin E1 polypeptide (Fig. 1D), which has traditionally been called the T or translocation domain. In this tradition, the T and R domains, respectively, are responsible for translocation across the periplasmic space and the outer membrane. In fact, the functional definition of the T (translocation) domain involves translocation across both the outer membrane and the periplasmic space, a definition distinct from the traditional view in which the three structurally definable colicin domains (R, T, and C) functioned in the three definable compartments of the cell envelope, outer membrane, periplasm, and cytoplasmic membrane, respectively.

3.2. The second receptor for colicin E1

The unique 100-Å extended 12-stranded antiparallel trimeric helical barrel structure of the TolC outer membrane protein has a 35-Å internal diameter channel that extends nearly through the 100-Å length [52], and therefore across most of the ca. 150-Å-wide periplasmic space. This wide channel tapers to a narrow opening on its periplasmic end, which might restrict the passage of a polypeptide substrate. However, because TolC is known to be utilized for the cellular export of large proteins (ca. 170 kDa) in type I secretion, it has been inferred that the tapered end of the trimeric helical barrel structure must open to allow passage of protein substrates that must be at least partially unfolded [53]. This would also imply that colicin E1 can be translocated through this channel across the outer membrane into the periplasmic space. Passage into the periplasm would occur with the T domain transported first and the colicin C domain translocated in a mostly unfolded state. This requirement was also inferred from the loss of cytotoxicity of disulfide cross-linked C domain of colicin A [54]. After passing through TolC, further translocation would be driven

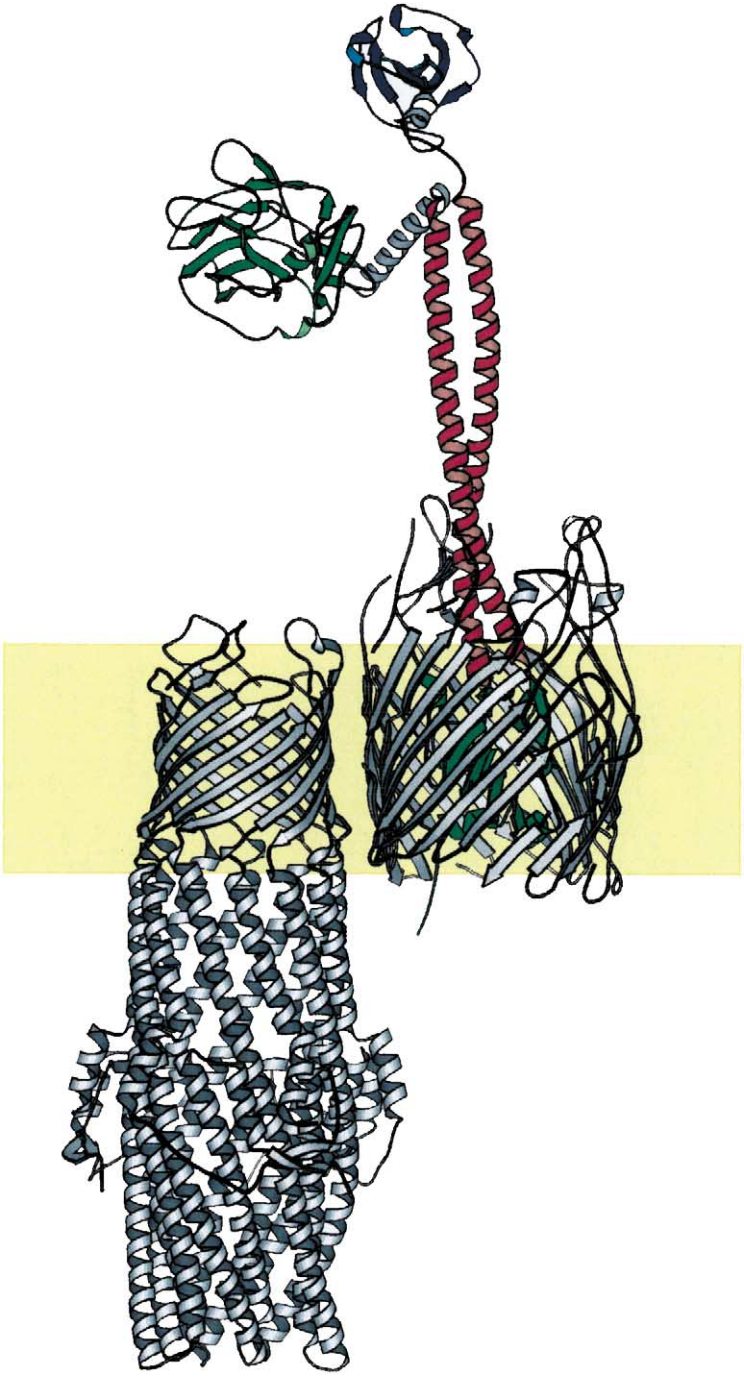

Fig. 9. Two-receptor concept: BtuB and Tol C outer membrane proteins used for uptake of colicin E1. X-ray crystal structures of colicin E3 [2], FepA [71], and TolC [52] were used to model the initial recognition of colicin E3 by BtuB, and its proposed subsequent interaction with TolC in the *E. coli* outer membrane. The BtuB outer membrane receptor is shown as a modified (N-terminal "plug" segments 59–70 and 96–117 deleted) structure of the FepA receptor [71], based on its sequence identity and significant structure homology with BtuB.

by the function of TolA, which is not understood. The literature implies that the requirement of TolC for trans-periplasmic translocation among A-type colicins is unique to colicin E1. In this respect, it is of interest that colicin E1 is also unique among these colicins (E1, E2, E3, A, K, and N) in remaining mostly active with respect to cytotoxicity when 234 residues were deleted (TolAΔM54–A287) from the 421-residue TolA protein [55]. This implies that when the trans-periplasmic TolC is used for colicin translocation from the outer membrane, TolA does not have to span the periplasm from the cytoplasmic membrane in order to participate in translocation. TolA must direct further translocation through TolC, which ultimately must include transfer of the C domain that will interact with the TolQ,R

translocation proteins embedded in the cytoplasmic membrane. The trypsin-reversal phenomenon in which trypsin added to the cellular exterior can reverse inhibition of active transport by the C domain inserted into the cytoplasmic membrane [8,56,57] implies that part of the colicin E1 molecule, presumably the R domain, remains in the exterior of the outer membrane while the channel domain acts to depolarize the cytoplasmic membrane. As it has been proposed that the opening of the distal end of the TolC channel involves a twisting of the α-helical barrel [52], it may be speculated that one active function of the R domain at the exterior is to unwind the extended coiled-coil in order to generate the "open state" of the distal end of the TolC channel.

3.3. Function of the first receptor

In the two-receptor model for colicin import through the outer membrane, the primary BtuB receptor has two functions: (i) initial binding and sequestering of the colicin. It has been proposed that the initial interaction between colicin E3 is hydrophobic, between the tip of the colicin R domain and a hydrophobic region at the top of the plug domain of BtuB [2]. However, this region is actually not very hydrophobic as it contains three basic residues within six residues on either side of the M383–A384 turn. Electrostatic interactions would seem appropriate for the initial attractive interactions that must guide the R domain to the receptor over large distances. (ii) It has been proposed [4] that the function of the R-domain coiled-coil, which appears not to be necessary for high affinity binding to the BtuB primary receptor, is to unwind after binding to the primary receptor and thus induce extensive or partial unfolding of the T and C domains and their binding to, and entry into, the neighboring secondary TolC receptor-translocator (Fig. 10). The energy required for unfolding could be derived in vitro from the energy of binding to the receptor, and in vivo as well from the proton electrochemical potential stored across the cytoplasmic membrane.

3.4. One or two receptors for the other colicins?

The TolC protein has such a uniquely useful structure for inter-membrane protein translocation and is used so ubiquitously that one wonders why it is not required by more colicins. A requirement for a second outer membrane protein is not known for the B-group TonB-requiring colicin Ia, or for the small A-group colicin N that uses OmpF. The minimum channel aperture of the 16-stranded anti-parallel OmpF β-barrel is 7 × 11 Å [58], which may not be large enough to allow passage of an unfolded polypeptide. On one hand, a role for OmpF in colicin translocation was implied by the decrease in the OmpA aperture in a G119D colicin-resistant mutant in loop L3 of OmpF [59,60]. On the other hand, immobilization of the L3 loop by Cys–Cys cross-linking did not cause a major decrease in the activity of colicins N and A [61]. From these data and earlier data on an exterior location of residues needed for colicin N/A cytotoxicity [62], it was proposed that after binding to OmpF, these colicins cross the outer membrane outside OmpF without using any Omp ([3]; also, see discussion in Ref. [40].)

The A-group colicins E3, E9, and A seem to require the outer membrane protein, OmpF, in addition to BtuB. It has been previously proposed that OmpF could act as a channel to carry the unfolded T domain of colicin A across the outer membrane to make contact with TolA [63]. A prediction of the "two-receptor model" is that the ion channel size of the OmpF proteins should be occluded by these colicins. Preliminary experiments show discrete channel closing of OmpF channels by colicin E3 (Eurokova, Zakharov, Rokitskaya, Antonenko, and Cramer, unpublished). It would be of interest to study the OmpF–colicin interaction by site-directed spin labeling, as was done in studies of the interaction of colicin B with its FepA receptor [64].

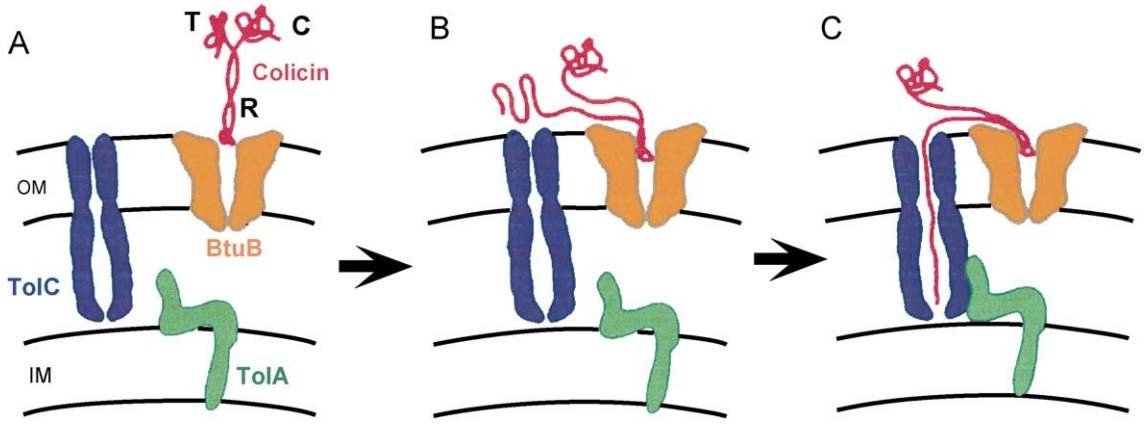

Fig. 10. Scheme of colicin E1 import: (A) initial recognition of R domain by BtuB receptor; (B) unwinding of extended coiled-coil of R domain would result in loss of domain interactions and partial unfolding of T-domain followed by its interaction with TolC; (C) TolA-mediated translocation of T-domain through TolC to inner membrane. IM, OM, inner (cytoplasmic) and outer membrane (modified from Ref. [4]). Bent conformation of TolA is non-canonical relative to the literature, and is based on its unique interaction with colicin E1 [55].

3.5. The functional R domain of colicin E3

Colicin E3 [65], and 135- and shorter peptides derived from its structurally defined R domain [5], bind to purified BtuB receptor with a 1:1 stoichiometry and high affinity. Far-UV CD and melting analysis show that the 135-residue peptide has a high content of α-helical coiled-coil, similar to the intact colicin E3. The helical coiled-coil content is reduced in a 60-residue "partial R" peptide, but restored upon binding. Thus, helical secondary and coiled coil tertiary structures appear to be necessary for high affinity binding. Growth of cells containing the BtuB receptor is inhibited by a 76-residue peptide of the putative R domain of colicin E9 [66] that has 92% sequence identity with the 135-residue R domain of colicin E3.

4. Epilogue

Based on the atomic structures of the pore-forming colicins (Section 1), studies on channel formation and the properties of the membrane-bound intermediate state carried out with synthetic membranes (Section 2), and structure–function of the outer membrane receptors and isolated R-domain peptides (Section 3), one may speculate briefly (and, of necessity, incompletely) on the in vivo pathway of colicin channel insertion into the cytoplasmic membrane.

(i) The colicin binds tightly to a receptor whose major function is recognition and binding, e.g., BtuB, for E-type colicins. (ii) A second proximal receptor, e.g., Tol C, translocates unfolded N- and C-terminal domains of colicin E1 across the outer membrane and most of the periplasmic space. (iii) These receptors and the Tol or Ton/Exb system may be concentrated in the region of inter-membrane adhesion zones. (iv) Unfolding at the initiation of translocation through outer membrane receptors is driven by unwinding of the R-domain coiled-coil. (v) The membrane surface potential and the network of TolA/TolQ/TolR, or TonB/ExbB/ExbD proteins mediate binding to the surface of the cytoplasmic membrane of the C-terminal channel domain in a conformationally flexible unfolded two dimensional helical array. (vi) During the binding process, some helical segments elongate to allow subsequent spanning of the inner membrane. (vii) Insertion into the cytoplasmic membrane is facilitated by conformational flexibility of the colicin bound to the surface of the cytoplasmic membrane, and specifically catalyzed by the integral membrane TolQ/TolR or ExbB/ExbD proteins.

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