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Analyzed by Disulfide Bond Engineering*

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Four colicin A double-cysteine mutants possessing a disulfide bond in their pore-forming domain were constructed to study the translocation and the pore formation of colicin A. The disulfide bonds connected α-helices 1 and 2, 2 and 10, 3 and 9, or 3 and 10 of the pore-forming domain. The disulfide bonds did not prevent the colicin A translocation through the Escherichia coli envelope. However, the mutated colicins were able to exert their in vivo channel activity only after reduction of their disulfide bonds. In vitro studies with brominated phospholipid vesicles and planar lipid bilayers revealed that the disulfide bond that connects the α-helices 2 and 10 prevented the colicin A membrane insertion, whereas the other double-cysteine mutants inserted into lipid vesicles. The disulfide bonds that connect either the α-helices 1 and 2 or 3 and 10 were unable to prevent the formation of a conducting channel in presence of membrane potential. These results indicate that α-helices 1, 2, 3, and 10 remain at the membrane surface after application of a membrane potential.

Colicin A is a bacteriocin that kills sensitive Escherichia coli cells by forming voltage-gated ion channels in cytoplasmic membranes. Like many toxins, colicin A is organized into structural domains, each of them carrying one function associated with the toxin's lethal activity (1). The N-terminal domain is involved in the translocation through the E. coli envelope, the central domain is responsible for the binding to a receptor on the bacterial surface, and the C-terminal domain possesses the pore-forming activity. The soluble form of this C-terminal domain obtained by mild proteolytic digestion was crystallized, and its three-dimensional structure was determined at 2.4 Å resolution. This molecule consists of a bundle of eight amphipathic α-helices surrounding two hydrophobic α-helices completely buried within the protein (2, 3).

In vitro, the pore formation is divided into several steps including a voltage-independent membrane insertion and a voltage-dependent channel opening (4). The first step is initiated by an electrostatic interaction between colicin A and negatively charged phospholipids (5–7). Two models have been proposed to picture the conformational changes required for the colicin membrane insertion. In the first model, called the "umbrella model," the three layers of helices that form the soluble structure are rearranged so that the hydrophobic hairpin of helices 8 and 9 traverses the membrane, whereas the helical pair 1 and 2 folds out on the surface in the opposite direction to helices 4–7 (8–10). In the second model, called the "penknife model," the hydrophobic helical hairpin lies parallel to the membrane plane (4, 11, 12).

Colicin channels inserted in planar lipid bilayers are opened by applying a trans-negative potential over a certain threshold voltage (13, 14). The voltage gating of colicins involves the translocation of parts of the protein across the membrane exposing different domains to the cis and trans solutions in the open and closed states (15, 16). Recently, Slatin et al. (17) have demonstrated that large domains of colicin Ia can reversibly flip across the membrane.

In a previous paper, we have discussed the effect of introducing four disulfide bonds in the colicin A pore-forming domain in order to analyze the voltage independent membrane insertion and the voltage dependent channel opening of the colicin A pore formation (4). These results suggested that the α-helices 8 and 9 did not cross the membrane without membrane potential and that the channel opening allowed drastic changes in the conformation of the pore-forming domain. In this paper we have used the same methodology to study the role of the first three helices of the colicin A pore-forming domain. The new double-cysteine mutants were tested for their capacity to promote K⁺ efflux from E. coli cells, to insert into lipid vesicles, and to form a conducting channel in planar lipid bilayers. The results obtained confirm the penknife model and suggest that the first three helices of the colicin A pore-forming domain are required to complete the colicin A translocation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media—The E. coli strain W3110 and plasmid pL1 have been described (4). The W3110 strain containing the various mutated plasmids was grown in LB medium and used for construction of the mutants. In order to measure the K⁺ efflux induced by the colicin’s activity, E. coli W3110 strain was grown at 37 °C to an A₅₆₀ of 0.5 (5 × 10⁶ cells/ml) in LB medium, washed, resuspended in 100 mM sodium phosphate buffer (pH 6.8), and kept on ice at a density of 5 × 10¹⁰ cells/ml.

DNA Manipulation—All single-cysteine mutants were obtained by the insertion of paired oligonucleotides into the plasmid pL1 between restriction sites (4) and were sequenced. The unique restriction sites used are listed in Table I. The double-cysteine mutants were constructed by fragment exchange between plasmids containing the single-cysteine mutants. The unique restriction sites and the plasmids used are listed in Table I.

Proteins Purification and Sulfhydryl Titration—Bacterial strains containing plasmids were induced with 300 nM of mitomycin C. All single or double-cysteine mutants were expressed at the same level as

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The restriction sites and the plasmids used to introduce cysteine residues in the α-helices of the colicin A C-terminal domain are indicated. The sequence of the C-terminal domain has been published previously (4).

| Plasmids | Mutations | Helices | Restriction sites |
|----------|-----------|---------|------------------|
| OH1 | 19C | H1 | XbaI/AvrII |
| OJ7 | A42C | H2b | AvrII/BspMI |
| PB7 | 19C/A42C | H1/H2 | AvrII/HindII from OJ 7 to OH1 |
| OJ4 | K33C | H2a | AvrII/BspMI |
| OP1 | E198C | H10b | Mlu/Eag |
| PA1 | K33C/E198C | H2/H10 | BstEII/HindII from OP1 to OJ 4 |
| OL3 | 166C | H3b | BspMI/Sfi |
| OM3 | A181C | H9 | Nhe/NarI |
| OQ3 | 166C/A181C | H3/H9 | BstEII/HindII from OM3 to OLM3 |
| OK3 | S62C | H3a | BspMI/Sfi |
| ON1 | A192C | H10a | Nar/Mlu |
| OR8 | S62C/A192C | H3/H10 | BstEII/HindII from ON1 to OK3 |

*HindII is a unique site of pcDA9 (41).

the wild type colicin A. Colicin A single-cysteine mutants and double-cysteine mutants were purified from the extracellular medium, and their thiol groups were titrated as described previously (4).

In Vivo Activity—In vivo activities of the colicins were determined by measuring the changes of the K⁺ concentration in the external medium with a K⁺ valinomycin-selective electrode as described previously (18). To increase the intracellular potassium concentration above 400 nmol/mg dry weight, the cells (2 × 10⁷/mL) were incubated for 5 min at 37 °C in 100 mM sodium phosphate buffer (pH 7.2) containing glucose (0.2%, w/v) and 0.5 mM KCl. The purified double-cysteine mutants were reduced with 5 mM DTT at 37 °C for 10 min in 50 mM Tris-HCl (pH 8.0) buffer and added to the E. coli cells at a multiplicity (number of colicins/cell) of 400. The lag times were calculated as described previously (19).

Insertion Kinetics of Colicin A into Phospholipid Vesicles—The quenching of the intrinsic fluorescence of colicin A by Br-DOPG vesicles was used to follow the membrane insertion kinetics of colicin A (20). The phospholipids were Sigma Type II-S soybean L-α-phosphatidylcholine. The hole was pretreated with a solution of phospholipids in decane (10 mg/mL). Membranes were then formed with the same solution by the brush technique. All conductance measurements were performed in 1 M NaCl, 10 mM Tris acetate (pH 4.0), and 2 mM CaCl₂. Known voltages were applied across the membrane, before colicin addition, to the cis side compartment under stirring, and the resulting current responses were measured. The trans compartment was connected to virtual ground. 50 ng/ml of wild type or double-cysteine mutants, treated with or without DTT, were added to the cis side of a lipid bilayer. Alternatively, 1 ng of protein could be added to record unitary currents.

RESULTS

Choice of Sites for Engineered Disulfides—Potential sites for cysteine residue pairs that might form disulfide bonds were selected using the method of Hazes and Dijkstra (23). Briefly, potential residue pairs were initially selected on the basis of appropriate Cβ-Cβ distances. Sulfur positions were generated for these residues, and it was checked whether certain stereochemical criteria were obeyed. A major criterion was that the α-β angles could not deviate by more than 30 ° from the observed preferences. Selected pairs were subjected to energy minimization and energetically favorable conformations were chosen (less than 10 kcal/mol) (Table II). Success of this method relies on the assumption that main chain conformations are very similar between wild type and mutant. Calculations were based on the refined model of the colicin A C-terminal domain (3). This model has been refined to an R-factor of 0.18 at 2.4 Å resolution with good stereochemistry.

The disulfide bonds were chosen to connect the first three helices of the colicin A C-terminal domain to the neighboring helices. These bonds, Ile⁵⁹ → Cys/Ala⁶⁰ → Cys (H1/H2), Lys⁵⁳ → Cys/Glu⁹⁸ → Cys (H2/H10), Ile⁶⁶ → Cys/Ala¹⁸¹ → Cys (H3/H9), and Ser⁶² → Cys/Ala¹⁹²→Cys (H3/H10) connected helices 1 and 2, helices 2 and 10, helices 3 and 9, and helices 3 and 10, respectively (Fig. 1). To introduce these disulfide bonds, we used the LR1 plasmid, which encodes the entire colicin A protein (4). From this plasmid, cassette mutagenesis was performed to introduce the cysteine residues (Table I). All the mutant proteins were expressed at the wild type level and were purified.

Disulfide Bond Formation—Disulfide bond formation of the double-cysteine mutants was analyzed by sulfhydrol titrations. The purified proteins were first denatured in 8 M urea in order to expose the buried thiol groups, and then they were incubated with or without a reducing agent such as DTT and finally incubated with a thiol-specific reagent monobromobimane. The thiol groups were titrated with the monobromobimane only after the reduction of the double-cysteine mutants by DTT (Fig. 2). The double-cysteine mutants that reacted with the monobromobimane migrated slower than either the wild type or the nonreactive double-cysteine mutants in a nonreducing 7% urea gel (Fig. 2). These results indicate that without a reducing agent the two thiol groups of the double-cysteine mutants form a disulfide bond.

The Disulfide Bonds Inhibit the in Vivo Colicin A Activity—The effect of the disulfide bonds on the colicin A pore-forming activity were tested in E. coli cells by using a K⁺/valinomycin selective electrode. It has been previously described that colicin A, when added to E. coli sensitive cells, induced an efflux of cytoplasmic K⁺ that was correlated with the activity of the pore. The rate of this K⁺ efflux saturated at about 400 mole/cell (18). At this multiplicity, the double-cysteine mutants did not cause a K⁺ efflux in contrast to the corresponding single-cysteine mutants (Table III). However, when these inactive mutants were preincubated with 5 mM DTT before their addition to the cells, their ability to induce a cytoplasmic K⁺ efflux was restored to the wild type level (Table III). This result indicates that the disulfide bonds block the in vivo colicin A pore-forming activity.

The Disulfide Bonds Do Not Prevent the Colicin A Translocation through the E. coli Envelope—Different steps can be blocked by the disulfide bonds. We examined the effect of the disulfide bond on the colicin A receptor on the bacterial surface and translocation through the E. coli envelope. The K⁺ efflux induced by colicin A is preceded by a lag that corresponds to the time required for its binding to BtuB and OmpF and for its translocation through the envelope (18). At a multiplicity of 400 and at 37 °C, the lag time equaled 27 s for the wild type colicin A and 29, 32, 28, and 30 s, respectively, for the H1/H2,
Interestingly, the time elapsing between the DTT addition and the in vitro (see above). It is likely that this time represents the time required for the colicins translocation at the multiplicity of 400 and at 37°C, the addition of 5 mM DTT equaled 20-425 s (data not shown). This time is shorter than the time required for the colicins translocation (see above). It is likely that this time represents the time needed for DTT action. Indeed, the time required for full in vitro reduction of the mutants treated with 5 mM DTT equaled 3-8 min (data not shown). Because colicin A is unfolded during its translocation, the time required for DTT action is shorter in vivo than in vitro (19, 24). This result indicates that the mutated colicins were fully translocated despite the presence of their disulfide bonds. The Voltage-independent Membrane Insertion of the Colicin A C-Terminal Domain (26) and favored its membrane insertion (4, 11). The ability of the H2/H10 mutant to insert into Br-DOPG vesicles. The H2/H10 and H3/H10 mutants were able to insert into Br-DOPG vesicles without preincubation with DTT (Fig. 3, bottom). The insertion kinetic constant and the residual fluorescence of the oxidized H3/H9 and H3/H10 mutants and reduced H2/H10 mutant were quite similar to those of the colicin A wild type (Table IV). However, the multiple kinetic constant of the oxidized H1/H2 mutant was higher compared with the wild type. A similar behavior was encountered previously with cysteine mutation, which destabilized the colicin A conformation (26) and favored its membrane insertion (4, 11). The Disulfide Bond That Connects Helices 2 and 10 Did Not Prevent the Ionic Channel Formation—The ability of the double-cysteine mutants to form functional ionic channels when incorporated in lipid bilayer was evaluated with the planar bilayer technique. As described previously (14), adding the wild type colicin A to the cis side of the bilayer resulted in voltage-dependent currents. The current activated at large depolarizations (e.g., +90 mV in Fig. 4A) and deactivated upon repolarization. Both activating and deactivating rates were strongly voltage-dependent. Once incorporated in a functional state, the channel opened and closed at a rate determined by the membrane polarization. The H1/H2 and H3/H10 mutants were found able to form functional channels without the need of preincubation with DTT (Fig. 4B). The overall behavior of the resulting macroscopic current was similar to that of the wild type colicin A. Unitary currents (insets in Fig. 4, A and B) of the wild type, H1/H2, and H3/H10 mutants had similar amplitude corresponding to a unitary conductance of 5.1 ± 0.6 pS. By contrast, the disulfide bonds connecting either helices 3 and 9 or helices 2 and 10 prevented the formation of functional channels, even in a permanently depolarized bilayer (first part of current trace...
in Fig. 4C). Preincubating the mutants with DTT or adding DTT to the mutant-containing cis side of the bilayer (Fig. 4C) restored the ability of the protein to incorporate in the bilayer. The resulting channel had a unitary conductance similar to that of wild type colicin A (inset in Fig. 4C). The voltage gating of the reduced H3/H9 and H2/H10 mutants differed from that of the wild type protein, with fast flickering in the open state and incomplete deactivation at large negative potentials (not illustrated).

**DISCUSSION**

Soluble pore-forming colicins undergo conformational changes during their membrane insertion. They are unfolded during their reception and translocation through the E. coli envelope (19, 24). In the presence of membrane potential, the colicin A C-terminal domain inserts into the membrane and forms a conducting channel (4, 17, 27, 28). In order to analyze the conformational changes associated with these different steps, four disulfide bonds were introduced in the colicin A C-terminal domain. The disulfide bonds were designed to connect the first three helices of the pore-forming domain to neighboring helices. The aim of these disulfide bonds was to restrict...
the degree of freedom of the first three helices of the colicin A C-terminal domain and to determine the influence of these helices in the colicin A translocation, membrane insertion, and/or channel formation.

In the first part of this paper we demonstrated that all the disulfide bonds were able to prevent the in vivo colicin A activity without affecting the colicin A translocation through the E. coli envelope. Indeed, the oxidized colicins were inactive, but as DTT was added in vitro, they recovered full channel activity as judged from their capacity to induce an efflux of cytoplasmic K⁺ of the same amplitude as the wild type colicin. Channel activity was also recovered by incubating the oxidized colicins with E. coli cells and then adding DTT. The translocation of the wild type colicin A and the mutants required 27–32 s. Interestingly, when the oxidized colicins were incubated with bacteria for a time longer than that needed for full translocation (see above), the efflux of K⁺ started only 8–10 s after DTT addition. This short delay, which represents the time needed for DTT action, indicated that the oxidized colicins were translocated and already close to the inner membrane when DTT was added to the external medium. This result is similar to that obtained with four other disulfide bonds connecting helices 1 and 9, helices 5 and 6, helices 7 and 8, and helices 9 and 10 (4, 19).

In the second part of this paper, we have studied the effect of the disulfide bonds on the colicin A membrane insertion and channel opening in vitro. The results confirm that the pore formation includes at least two steps: a voltage-independent membrane insertion and a voltage-dependent channel opening. The disulfide bond that connects helices 3 and 9 did not prevent the colicin A membrane insertion but blocked the channel opening. The same result has been previously described with the disulfide bonds connecting helices 5 and 6, helices 7 and 8, and helices 9 and 10, respectively (4).

In the following discussion, we will first analyze the implication of this work on the voltage-independent membrane insertion of colicin A into the membrane and how this is compatible with the umbrella model described by Parker et al. (3, 8) and supported by experiments on colicin E1 (9, 10, 27, 29, 30) or with the penknife model proposed by Lakey et al. (11) and supported by experiments on colicin A (12, 31, 32).

The fluorescence quenching assay with brominated lipid vesicles has been analyzed in detail by González-Mañas et al. (20, 21). This method detects the actual membrane insertion and not the adsorption of colicin to the vesicle surface. The charged amphipathic helices 3–7, which contain the three tryptophan residues of the pore-forming domain of colicin A, most probably sit in the membrane with the apolar region facing the hydrophobic core of the bilayer, exposing the fluorophores to the bromine, and the polar face in contact with the solvent protecting the tryptophans from soluble quenchers (21, 33).

The two models considered that the voltage-independent membrane insertion is driven by the opening of the molecule extending helices 1 and 2 away from the other helices. In agreement with these models, locking helices 2 and 10 together prevents the colicin A membrane insertion, whereas locking helices 1 and 2 and helices 3 and 10 has no measurable effect on membrane insertion. The result obtained with the disulfide bond connecting helices 3 and 9 supports the penknife model. Indeed, the umbrella model predicts a large distance increase between helix 3 and 9 upon membrane insertion. This is not compatible with the ability of the H3/H9 mutant to insert into the membrane. In contrast, the penknife model suggests that the hydrophobic helices 8 and 9 remain close together with helices 3–7 and helix 10 upon membrane insertion. Our results confirm those obtained with the disulfide bonds previously introduced in the colicin A C-terminal domain. Indeed, the disulfide bonds that connect helices 1 and 9 prevented the colicin A membrane insertion, whereas the disulfide bonds that connect helices 5 and 6, helices 7 and 8, and helices 9 and 10 did not (4).

In the presence of membrane potential, some helices of the colicin C-terminal domain insert into the membrane and form a conducting channel. Many studies have focused on the identification of the helices forming the open state of the channels (17, 26, 29, 34). On the basis of biochemical labeling experiments, Merril and Cramer have proposed that the colicin E1 channel was formed by four helices, the hydrophobic helical hairpin and an amphipathic helical hairpin composed by helices 5 and 6 (29). This amphipathic helical hairpin has been identified as the voltage-sensitive segment. Subsequent experiments have confirmed this result (27, 35). Recently, Slatin et al. (17) have demonstrated that at least 31 amino acids of the colicin Ia C-terminal domain were translocated across the membrane after application of a trans-negative potential. This result indicates that the voltage gating is not only associated with the insertion of a helical hairpin into the membrane but also with the translocation of part of the protein across the membrane.

The results obtained in this paper suggest that helices 1, 2, 3, and 10 remain at the membrane surface after application of a trans-negative voltage. Indeed, the disulfide bonds that connected helices 1 and 2 and helices 3 and 10 did not prevent the membrane insertion or the channel opening. In contrast, the disulfide bond between helices 3 and 9 did not prevent the membrane insertion but prevents the channel opening, suggesting that helix 9 should move away from helix 3 upon channel opening by the membrane potential. Probably, the helical hairpin (helices 8 and 9) becomes perpendicular to the plane of the lipid bilayer. The same interpretation could be given with the results obtained with the disulfide bonds connecting helices 9 and 10 and helices 7 and 8 (4) and could suggest that helix 7 is also at the membrane surface. Nevertheless, we cannot rule out the possibility of helix 7 forming part of the channel. Taking together these results, we can postulate that the open state of the colicin A channel is formed by four helices, helices 8 and 9 and helices 5 and 6.

It has been previously described that the first three helices of the colicin A C-terminal domain were not involved in ion conduction through the channel (36). According to this result, the two disulfide bonds connecting helices 1 and 2 and helices 3 and 10 did not prevent the in vitro channel opening of the toxin. However, these disulfide bonds blocked the in vivo activity of the colicin A without affecting its translocation through the E. coli envelope. Previous results have indicated that colicin A and Ia remained in contact with the outer membrane when they formed a channel in the inner membrane of sensitive cells (24, 37–39). In order to explain the results obtained with the disulfide bonds connecting helices 1 and 2 and helices 3 and 10, we can speculate that helices 1, 2, and 3 span the periplasm of E. coli and connect the central domain of the toxin fixed to the outer membrane to helices 4–9 of the C-terminal domain inserted into the inner membrane.

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