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Membrane Topology of the Colicin A Pore-forming Domain 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 pLR1 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 A_{650} of 0.5 (5×10^8 cells/ml) in LB medium, washed, resuspended in 100 mM sodium phosphate buffer (pH 6.8), and kept on ice at a density of 5×10^{10} cells/ml.

DNA Manipulation—All single-cysteine mutants were obtained by the insertion of paired oligonucleotides into the plasmid pLR1 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 ng/ml of mitomycin C. All single or double-cysteine mutants were expressed at the same level as

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TABLE I
Cysteine mutants

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	I19C	H1	*Xba*I/*Avr*II
OJ7	A42C	H2b	*Avr*II/*Bsp*MII
PB7	I19C/A42C	H1/H2	*Avr*II/*Hin*dIII from OJ7 to OH1[a]
OJ4	K33C	H2a	*Avr*II/*Bsp*MII
OP1	E198C	H10b	*Mlu*I/*Eag*I
PA1	K33C/E198C	H2/H10	*Bst*EII/*Hin*dIII from OP1 to OJ4[a]
OL3	I66C	H3b	*Bsp*MII/*Sfi*I
OM3	A181C	H9	*Nhe*I/*Nar*I
OQ3	I66C/A181C	H3/H9	*Bst*EII/*Hin*dIII from OM3 to OL3[a]
OK3	S62C	H3a	*Bsp*MII/*Sfi*I
ON1	A192C	H10a	*Nar*I/*Mlu*I
OR8	S62C/A192C	H3/H10	*Bst*EII/*Hin*dIII from ON1 to OK3[a]

[a] *Hin*dIII is a unique site of pColA9 (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 (2×10^9/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[1] 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 synthesis of Br-DOPG, the vesicle preparations, and the fluorescence measurements were carried out as described previously (4, 20, 21).

In Vitro Activity—Planar bilayers were formed in a small hole connecting two aqueous compartments using the Mueller-Rudin technique (22). 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), 2 mM $CaCl_2$. 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 χ^{-3} 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.

[1] The abbreviations used are: DTT, dithiothreitol; Br-DOPG, brominated dioleoylphosphatidylglycerol.

TABLE II
Predicted geometries of engineered disulfides in colicin A

Colicin A pore-forming domain crystallizes as two monomers in the asymmetric unit of the unit cell. Calculations were performed on both. χ^{-3} is the Cβ1-Sγ1-Sγ2-Cβ2 dihedral angle. Energy of the disulfide bond is determined as defined by Hazes and Dijkstra (23).

Mutants	Monomer A		Monomer B	
	χ^{-3}	Energy	χ^{-3}	Energy
	degrees	*kcal/mol*	*degrees*	*kcal/mol*
H1/H2	−72	3.4	−86	3.5
H2/H10	126	5.1	135	5.8
H3/H9	−93	2.7	−105	3.3
H3/H10	130	5.9	126	5.7

The disulfide bonds were chosen to connect the first three helices of the colicin A C-terminal domain to the neighboring helices. These bonds, $Ile^{19} \rightarrow Cys/Ala^{42} \rightarrow Cys$ (H1/H2), $Lys^{33} \rightarrow Cys/Glu^{198} \rightarrow Cys$ (H2/H10), $Ile^{66} \rightarrow Cys/Ala^{181} \rightarrow Cys$ (H3/H9), and $Ser^{62} \rightarrow Cys/Ala192 \rightarrow Cys$ (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 sulfhydryl 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 M 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 molecules/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 reception 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,

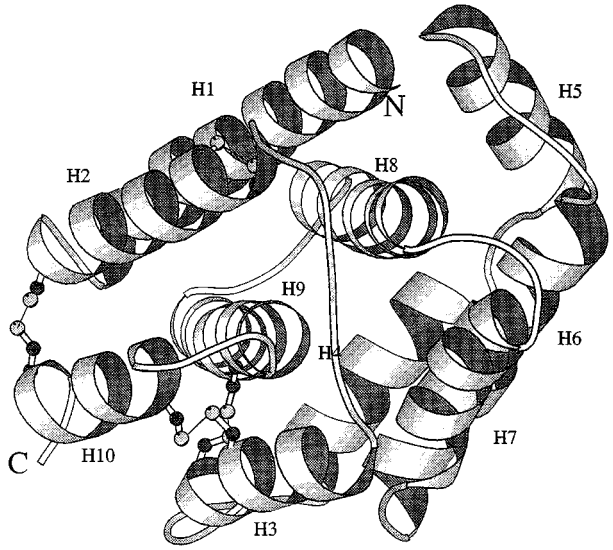

FIG. 1. **Positions of the disulfide bonds in the colicin A C-terminal domain.** Ribbon plot of the colicin A C-terminal domain displaying the disulfide bonds. The 10 α-helices are numbered H1 through H10. Coordinates of the colicin A C-terminal domain are deposited in the Brookhaven Protein Data Bank (ICOL). This figure was produced by the computer program MOLSCRIPT (40).

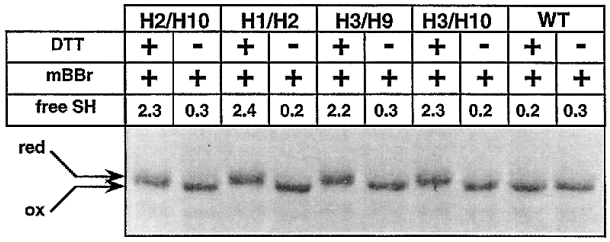

FIG. 2. **Sulfhydryl titrations and electrophoretic migration of double-cysteine mutants.** Purified colicins (2 mg/ml) were denatured in 8 M urea and incubated with or without 25 mM DTT at 37 °C for 4 h, and then the sulfhydryl contents (*free SH*) were determined by reacting the denatured colicins with a 5-fold molar excess of monobromobimane for 2 h at room temperature. Colicin samples (3 μg) were run on a 7% SDS-polyacrylamide, 7 M urea gel (lacking reducing agent). The *arrows* indicate the position of the reduced (*red*) *versus* oxidized (*ox*) disulfide colicins. *WT*, wild type.

H2/H10, H3/H9, and H3/H10 mutants whose disulfide bonds were reduced before their addition to the cells (data not shown). After incubation of the oxidized colicins with cells during 1 min at the multiplicity of 400 and at 37 °C, the addition of 5 mM DTT resulted in a K^+ efflux whose initial rate was identical to that of the colicin mutant reduced *in vitro* (data not shown). Interestingly, the time elapsing between the DTT addition and the K^+ efflux equaled 8–10 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 Is Only Blocked by the Disulfide Bond That Connects Helices 2 and 10—The ability of the disulfide bonds to prevent the colicin A membrane insertion was tested with brominated phospholipid vesicles. Liposomes made of brominated phospholipids are an efficient system for studying colicin A membrane

TABLE III
In vivo activities of the cysteine mutants

The ability to induce a K^+ efflux of the purified cysteine mutants and colicin A wild type, preincubated with or without 5 mM DTT was investigated on the *E. coli* W3110-sensitive strain at the multiplicity of 400 molecules/cell. The initial rate of K^+ efflux was determined in the linear part of the K^+ efflux curve as previously described (18).

Mutants	Initial rate of K^+ efflux	
	−DTT	+DTT
	nmol min^{-1} mg^{-1}	
Wild type	400	410
H1	390	420
H2b	380	370
H1/H2	9	320
H2a	370	380
H10b	390	380
H2/H10	15	335
H3b	410	390
H9	420	410
H3/H9	20	425
H3a	390	400
H10a	400	380
H3/H10	15	400

insertion. Indeed, the bromine atoms located at the midpoint of the phospholipid acyl chain quench the intrinsic fluorescence of the inserted colicin A (20, 21). The quenching is due to a collisional mechanism or to a short distance resonance energy transfer (25). From these quenching experiments, the insertion kinetic constant (k) and the residual tryptophan fluorescence (RF) can be calculated (4).

The disulfide bond that connects helices 2 and 10 was the only one able to prevent the colicin A membrane insertion (Fig. 3, *top*). Reduction of the disulfide bond with DTT restored the ability of the H2/H10 mutant to insert into Br-DOPG vesicles. The H1/H2, H3/H9, 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 insertion kinetic constant of the oxidized H1/H2 mutant was higher as 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 Connected Helices 1 and 2 and Helices 3 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. 4*A*) 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. 4*B*). 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

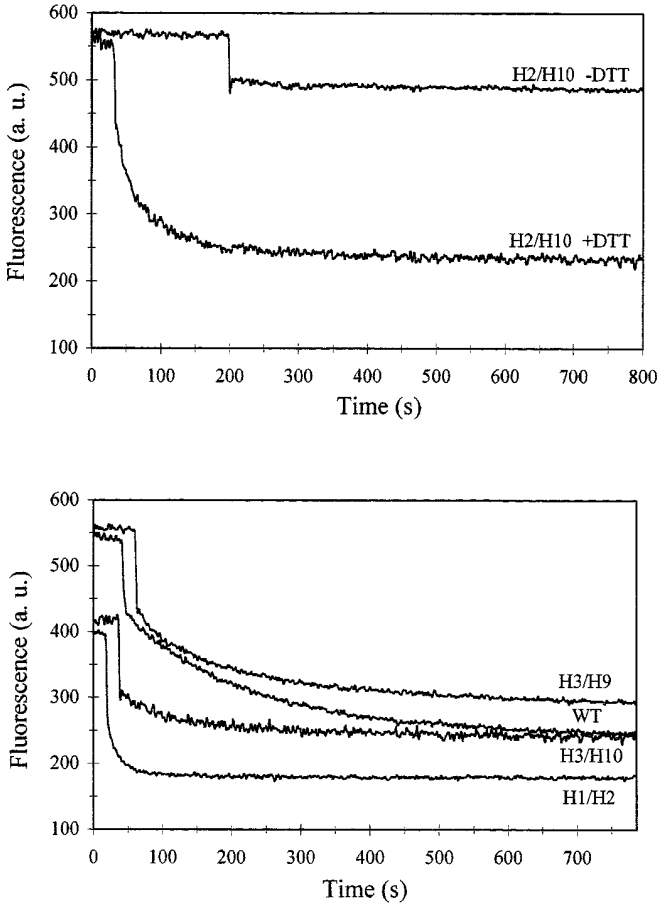

FIG. 3. **Kinetics of the quenching of the intrinsic fluorescence of double-cysteine mutants and wild type colicin A incubated with or without DTT by Br-DOPG vesicles.** To 400 μl of a colicin solution, 25 μg/ml in 50 mM Tris acetate buffer (pH 5.0) pretreated with or without 25 mM DTT were added 20 μl of a 10 mg/ml vesicle suspension in the same buffer (R_i = 450), and the tryptophan emission fluorescence was recorded as a function of time. Spectra are normalized to give identical fluorescence at t = 0.

TABLE IV
Insertion data of the cysteine mutants and wild type colicin A

The residual fluorescence (*RF*) of each protein, untreated or treated with DTT, inserted into Br-DOPG vesicles were calculated as previously described (4).

Mutants	Insertion rate constant		Residual fluorescence	
	−DTT	+DTT	−DTT	+DTT
	$(k)\ s^{-1}$		F_∞/F_0	
Wild type	0.0113	0.0134	0.46	0.43
Thermo (wild type)	0.0175	0.0166	0.15	0.19
H1/H2	0.0745	0.0652	0.47	0.50
H2/H10	No insertion	0.0364	0.85	0.45
H3/H9	0.0158	0.0142	0.55	0.48
H3/H10	0.0166	0.0125	0.58	0.55

in Fig. 4*C*). Preincubating the mutants with DTT or adding DTT to the mutant-containing *cis* side of the bilayer (Fig. 4*C*) 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. 4*C*). This process was, however, slow, and several depolarizations were required for the mutants to induce large currents. Although we did not performed a systematic study, it appeared that 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

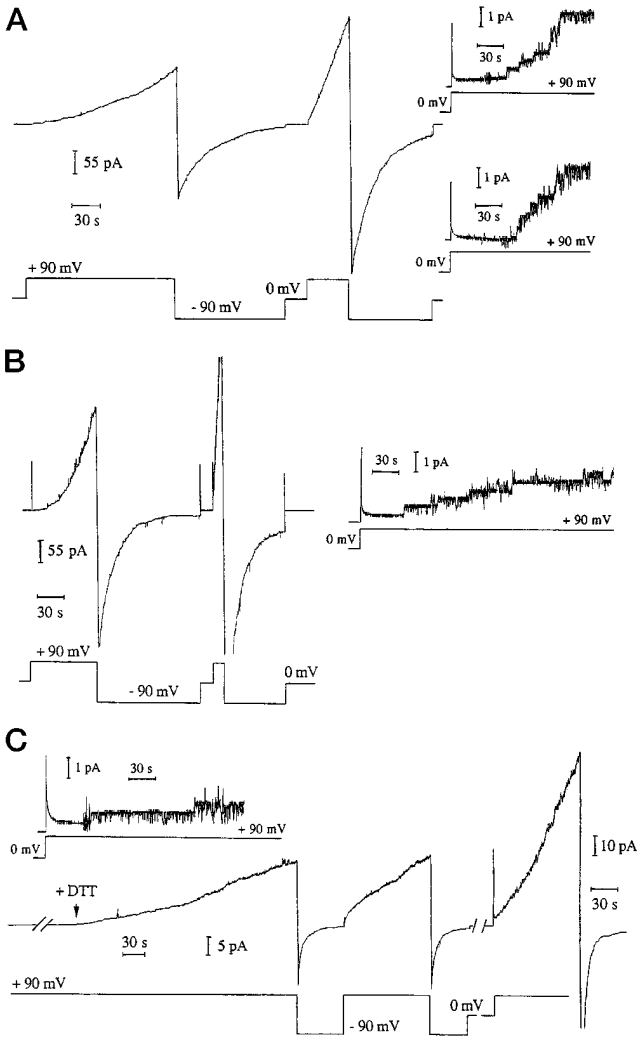

FIG. 4. **Pore-forming activity of double-cysteine mutants incorporated in planar lipid bilayers.** *A*, control recording of the current generated by the wild type protein. The first depolarizing step to +90 mV induced a slowly rising current, whereas the current in the second +90 mV step had a fast onset (see text for details). The current deactivated upon steping back to −90 mV. *Inset*, unitary currents under control conditions (*upper trace*) and after incubating the protein with DTT (2 mM, 30 min, 30 °C). *B*, current induced by the oxidized H1/H2 mutant subjected to the same voltage program as in *A*. *Inset*, unitary currents resulting from the opening of channels formed by the oxidized H1/H2 protein. *C*, behavior of the oxidized H3/H9 mutant in response to a +90 mV step. The oxidized mutant was inactive (*left-hand part* of the current trace). Adding DTT (2 mM) to the *cis* compartment induced an outward current at +90 mV whose amplitude and activation rate increased with time (*right-hand part* of the recording). The current deactivated at −90 mV. *Inset*, unitary currents from DTT (2 mM)-treated H3/H9 mutant.

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, Merill 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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