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Colicin A unfolds during its translocation in *Escherichia coli* cells and spans the whole cell envelope when its pore has formed

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The addition of the pore forming colicin A to *Escherichia coli* cells results in an efflux of cytoplasmic potassium. This efflux is preceded by a lag time which is related to the time needed for the translocation of the toxin through the envelope. Denaturing the colicin A with urea, before adding it to the cells, did not affect the properties of the pore but decreased the lag time. After renaturation, the lag time was similar to that of the native colicin. This suggests that the unfolding of colicin A accelerates its translocation. The addition of trypsin, which has access neither to the periplasmic space nor to the cytoplasmic membrane, resulted in an immediate arrest of the potassium efflux induced by colicins A and B. The possibility that trypsin may act on a bacterial component required for colicin reception and/or translocation was ruled out. It is thus likely that the arrest of the efflux corresponds to a closing of the pores. This long distance effect of trypsin suggests that part of the polypeptide chain of the colicins may still be in contact with the external medium even when the pore has formed in the inner membrane.

Key words: colicin A/pore closing/translocation/trypsin/unfolding

Introduction

Polypeptides which are transported across membranes usually need to be devoid of a stable tertiary structure (Randall and Hardy; 1986; Eilers *et al.*, 1988). To maintain such a competent conformation for the translocation, several cytosolic or membranous factors may be required (Meyer, 1988). This rule appears to apply both to the import across the mitochondrial envelope (Verner and Schatz, 1988; Hartl *et al.*, 1989) and the endoplasmic reticulum membrane, and to the export across the bacterial cytoplasmic membrane (for a review see Saier *et al.*, 1989).

Although much attention has been focused on the export processes in bacteria, very little is known about the less usual import of macromolecules across the bacterial envelope. Translocation of the double-stranded DNA of phage through the *Escherichia coli* envelope involves the participation of phage proteins forming pores in the inner membrane (Boulanger and Letellier, 1988, 1992; Feucht *et al.*, 1990). Furthermore, this translocation appears to take place in the sites of contact between the inner and outer membranes (Guihard *et al.*, 1992), the isolation of which has been described by Ishidate *et al.* (1986).

To kill sensitive *E.coli* cells, colicins must also be imported. The toxins first bind to a specific receptor located on the surface of the outer membrane; they are then translocated through the envelope to their target (the inner membrane or the cytoplasm) on which they finally exert their killing activity (for a review see Lazdunski *et al.*, 1988). The translocation process involves the participation of bacterial proteins encoded by the *tol A, B, C, Q, R* or *tonB* genes (Davies and Reeves, 1975a,b). Colicins are organized in three functional domains: the C-terminal domain carries the enzymatic or pore forming activity, the central domain is involved in the recognition of the receptor and the N-terminal domain is required for the translocation of the toxin (for reviews see Lazdunski *et al.*, 1988; Pattus *et al.*, 1990).

Colicin A belongs to the pore forming group of colicins. The pore activity of colicins was first inferred from *in vitro* experiments (Schein *et al.*, 1978; Pattus *et al.*, 1983) and from indirect *in vivo* observations (Konisky, 1982; Cramer *et al.*, 1983). We recently analysed the kinetics of the efflux of cytoplasmic potassium induced by colicin A in whole cells and showed that the toxin forms voltage-dependent channels in the inner membrane. This potassium efflux is preceded by a lag time of a few tens of seconds which corresponds to the time required for colicin A to bind to its receptor and to be translocated through the envelope (Bourdineaud *et al.*, 1990). Under defined conditions, it is possible to discriminate between these two steps and thus to analyse the dynamics of translocation independently of the binding. In this paper, we show that a pretreatment of colicin A with urea accelerates its transfer through the envelope, suggesting that it may translocate in an unfolded state. The finding that trypsin is able *in vivo* to induce the closure of the colicin A and B pores under conditions where the protease has access neither to the periplasmic space nor to the cytoplasmic membrane further suggests that these colicins maintain an extended conformation and are still in contact with the external medium even when their pore has formed in the inner membrane.

Results

In vitro denaturation and renaturation of colicin A

Incubation of colicin A with increasing concentrations of urea resulted in a shift of the tryptophan fluorescence emission maximum from 340 to 355 nm, which is characteristic of a change in their environment. Figure 1 shows that the fluorescence intensity ratio (340/355 nm) decreased continuously with increasing concentrations of urea from 2 to 8 M which suggests that urea affects the conformation of colicin A. Circular dichroism spectra of native and 8 M treated colicin A were performed: after the addition of 8 M

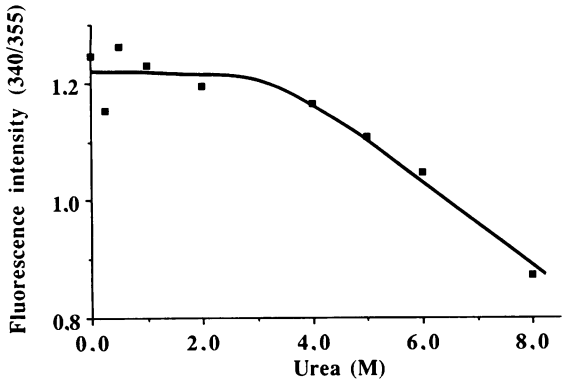

Fig. 1. Urea denaturation of colicin A as monitored by fluorescence emission spectroscopy: colicin A (final concentration, 3.7 μg/ml) was preincubated with variable concentrations of urea for 30 min at 22°C. The fluorescence emission spectra were monitored at λext = 280 nm and the denaturation curve was deduced from the ratio between the fluorescence intensities at 340 and 355 nm.

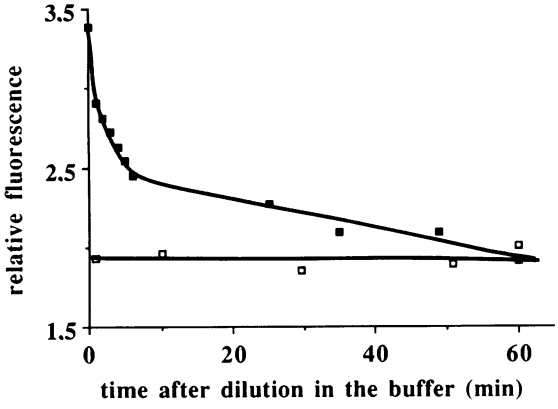

Fig. 2. Kinetics of the refolding of colicin A as determined from the fluorescence of the tryptophans: colicin A, denatured in 8 M urea, was diluted 200-fold in 115 mM NaCl. Aliquots (3.7 μg/ml) were taken and the fluorescence intensity at 355 nm (λext = 280 nm) was recorded at various times after dilution. (□) and (■) correspond to the native and urea-treated colicin A, respectively.

urea the secondary structure of colicin A was totally lost which indicates that colicin A was entirely denatured (data not shown).

The kinetics of renaturation of colicin A were determined by assaying the intrinsic fluorescence of the tryptophans. Denatured colicin A was diluted 200-fold in 115 mM NaCl and the fluorescence was measured at various times after dilution. Figure 2 shows that the renaturation is a biphasic process: the initial fast step ($\tau_{1/2}$ < 1 min), probably corresponding to an intradomain reorganization, was followed by a slow step and within ~50 min colicin A had recovered its native conformation. The renaturation of colicin A was confirmed by circular dichroism: a spectrum similar to the one of the native form was recovered (21% of α-helix, 20% β-sheet) when urea-treated colicin A was incubated for 1 h in sodium phosphate buffer (data not shown).

The kinetics of renaturation of colicin A were also assayed from protease accessibility experiments. Colicin A, either native or denaturated in 8 M urea, was diluted in sodium phosphate buffer and digested with thermolysin at various times after dilution. Figure 3 represents the immunodetection patterns of the colicin A fragments: as renaturation

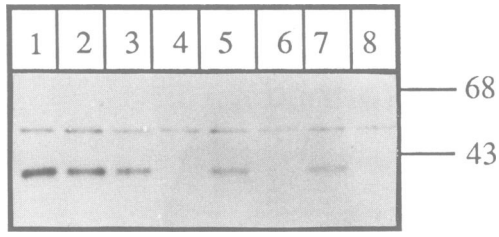

Fig. 3. Digestion profiles of native and renatured colicin A: the experiments were performed as described in Material and methods. Lanes 2, 3, 5 and 7 correspond to: 5, 10, 20 and 30 min of renaturation before thermolysin digestion respectively; in lane 1 denatured colicin A was digested immediately after dilution in the buffer (final urea concentration = 20 mM); in lanes 4, 6 and 8 native colicin A was incubated in 20 mM urea for 10, 20 and 30 min respectively before the digestion. Colicin A was detected with monoclonal antibodies. Molecular mass markers are indicated on the right.

proceeded, the urea-treated colicin A was found to recover a quite similar pattern of proteolysis to those of the native colicin A. These kinetics data are in good agreement with those obtained by fluorescence.

Translocation of colicin A is faster when the polypeptide chain is not in a native conformation

Adding colicin A to *E.coli* cells results in an efflux of cytoplasmic K^+; this efflux, which is due to the opening of the pores in the inner membrane (Bourdineaud *et al.*, 1990), increased with the number of added colicin molecules and saturated at a multiplicity of ~300 colicin A molecules per cell (Figure 4A). This efflux was preceded by a lag time corresponding to the time needed for the binding and the translocation of colicin A to occur. Figure 4B shows that this lag time decreased with increasing amounts of colicin A and became virtually constant beyond a multiplicity of 30. Since increasing the multiplicity increases the probability of interaction occurring between colicin A and its receptor, it is reasonable to suppose that beyond a multiplicity of 30, the time required for the adsorption will be small in relation to the time needed for the translocation. Consequently, lag time measurements at multiplicities >30 will provide a good approximation of the time needed for the translocation.

The initial rate of K^+ efflux and the lag time before the efflux were determined for various multiplicities of the native and 'renatured' colicin A. For this purpose, urea treated colicin A was diluted either by simply adding it to the cells or 30 min in advance. These times were chosen in view of the kinetics of renaturation of colicin A (see Figure 2) and thus corresponded to different stages of folding of colicin A. Urea, the concentration of which was 40 mM after dilution, had no effect on the response of the electrode. At all multiplicities, the 'renatured' colicin A showed a shorter lag time than the native colicin A; furthermore, after 30 min of renaturation, the lag time was intermediate between that of the native colicin A and that of the urea-treated colicin A which was diluted when added to the cells (Figure 4B). On the other hand, the initial rate of K^+ efflux was the same whatever the conformational state of colicin A (Figure 4A), and the membrane potential threshold at which the pore was closed (Bourdineaud *et al.*, 1990) was the same with both the native and 'renatured' colicin A (80 mV negative inside) (data not shown); this suggests that the number of channels opened in the cytoplasmic membrane

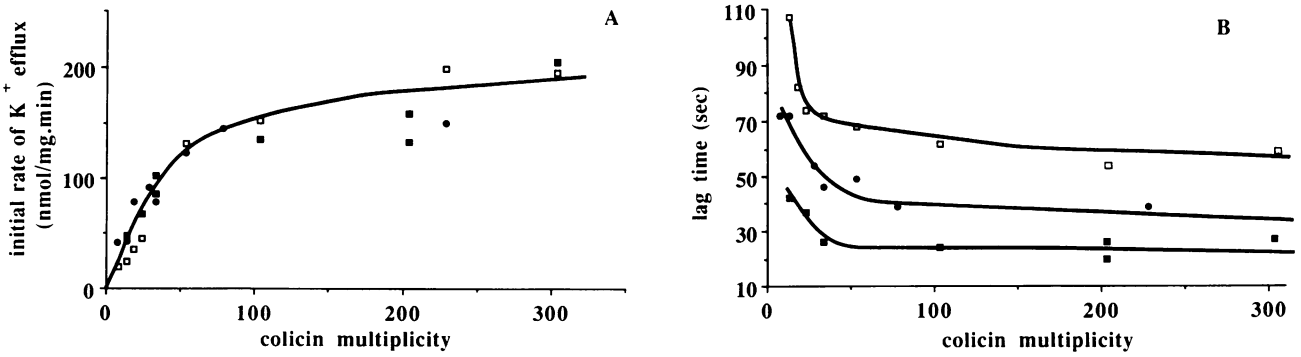

Fig. 4. Effects of the state of colicin A (native or renatured) on the initial rate of potassium efflux and on the lag time before the efflux. (**A**) the initial rate of potassium efflux was determined from the linear part of the efflux curve as previously described (Bourdineaud *et al.*, 1990). (□) and (●) represent the rates of efflux of native and renatured (30 min) colicin A, respectively. In (■) urea-treated colicin A was diluted when added to the cells. (**B**) the lag time is defined as the time elapsing between colicin addition and the onset of potassium efflux. Symbols are identical to those used in (A).

was the same in all cases and that the general characteristics of the pore are not modified by the prior denaturation of colicin A.

Urea-treated colicin A (multiplicity of 30) was first renatured for various periods and then added to the cells; Figure 5 shows that the lag time increased with the renaturation time and reached the lag time of the native colicin A after 60 min of renaturation.

The steps involving the binding of colicin A to its receptors BtuB or OmpF could not be by-passed, since urea-treated colicin A did not induce an efflux of K^+ in $BtuB^-$ or $OmpF^-$ cells. The translocation step could also not be by-passed since TolA and TolB mutants were inactive against urea-treated colicin A (data not shown).

Colicin A is still accessible to the external medium when its pore opens in the cytoplasmic membrane

Figure 6A shows the effect of trypsin on colicin A-induced K^+ efflux. In the absence of colicin, trypsin had no effect on the K^+ content of the cells. When trypsin was added 1 min after the onset of the K^+ efflux, this efflux stopped immediately. It is likely that the arrest of the efflux corresponds to the closing of only those pores inserted in the inner membrane. Indeed, 30 colicin A molecules were added per bacteria, a multiplicity at which the rate of efflux is proportional to the number of colicins and thus to the number of open pores (Figure 4A and Bourdineaud *et al.*, 1990). Furthermore since trypsin was added at a time where the rate of K^+ efflux is constant, it is likely that all colicin molecules have reached their target in the inner membrane. When *E.coli* cells were first treated with trypsin, then with trypsin inhibitor and a few minutes later with colicin A, the efflux of potassium occurred as normal (Figure 6A). This indicates that trypsin had not damaged an essential bacterial component involved in the binding or the translocation of colicin A.

Figure 6B shows that the pore formed by colicin B, which has neither the same receptor nor the same translocation machinery as colicin A (Roos *et al.*, 1989) also closed as soon as trypsin was added.

Since the pore formed *in vivo* by colicin A closes below a membrane potential threshold of 80 mV (negative inside) (Bourdineaud *et al.*, 1990) we checked whether a pretreatment of the cells with trypsin (see Material and methods) had any effect on the membrane potential. This possibility was ruled out since the membrane potential was 165 mV

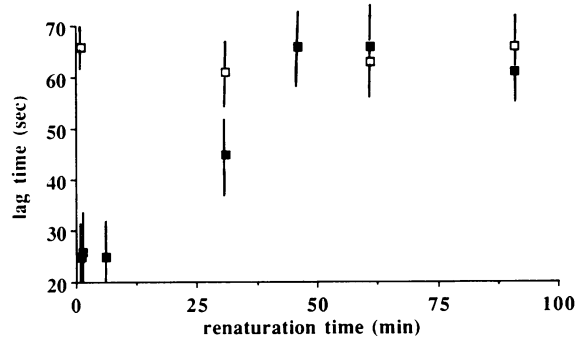

Fig. 5. Effects of the time of renaturation of colicin A on the lag time before the potassium efflux: colicin A, denatured in 8 M urea, was diluted 200-fold in 0.15 M NaCl and allowed to renature at room temperature for various times before being added to the cells. The lag time was determined as described above. The colicin multiplicity was 30. (□), native colicin A kept in 0.15 M NaCl containing 40 mM urea for the times indicated in the figure; (■), renatured colicin A. Each value of the lag time is the result of three to six separate experiments.

and 160 mV in the absence and in the presence of trypsin respectively.

Trypsin is known to have no access to the periplasmic space in the absence of a Tris−EDTA pretreatment (Tommassen and Lugtenberg, 1984). In these efflux experiments, the possibility cannot be ruled out, however, that the addition of colicin A may have disturbed the envelope in such a way that trypsin may have gained access to the periplasmic space. In order to determine whether or not this was the case, we checked for any degradation of the OmpA protein which can be proteolysed by trypsin only from the periplasmic side (Tommassen and Lugtenberg, 1984). Figure 7 shows that in the absence of colicin A, proteolysis of OmpA occurs only when the cells have been first permeabilized to trypsin by a Tris−EDTA treatment. The addition of colicin A to the cells under conditions known to arrest the K^+ efflux did not result in any cleavage of OmpA, which suggests that the trypsin was acting only on the external side of the outer membrane.

The pattern of degradation by trypsin of ^{35}S-labelled colicin A, incubated or not with cells, is shown in Figure 8. In the absence of trypsin, colicin A appeared as a unique band (63 kDa). When trypsin was added to bacteria previously treated for 1.5 min with colicin A (i.e. conditions

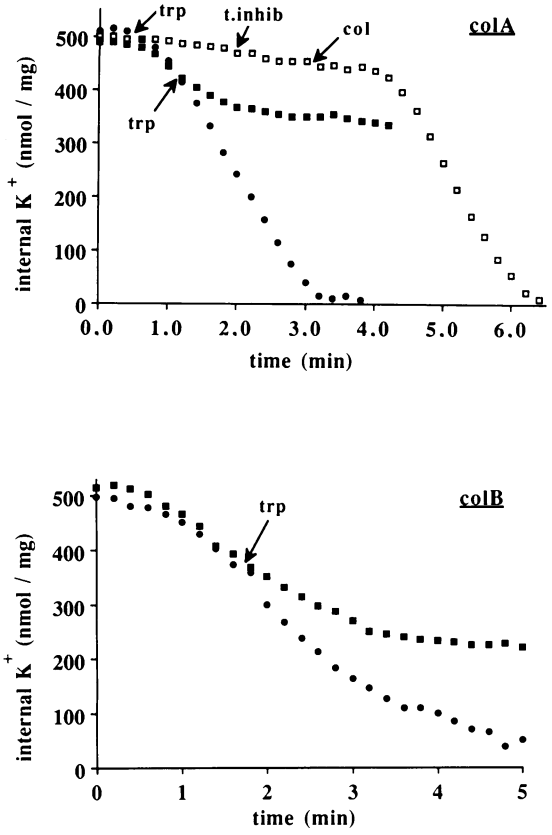

Fig. 6. (**A** and **B**) effects of trypsin on the potassium efflux induced by colicins A and B: trypsin was either added after the onset of potassium efflux (■) or before colicin (□); control without trypsin (●). Colicin multiplicity was 30. (trp: trypsin, 250 μg/ml; t.inhit: trypsin inhibitor, 120 μg/ml).

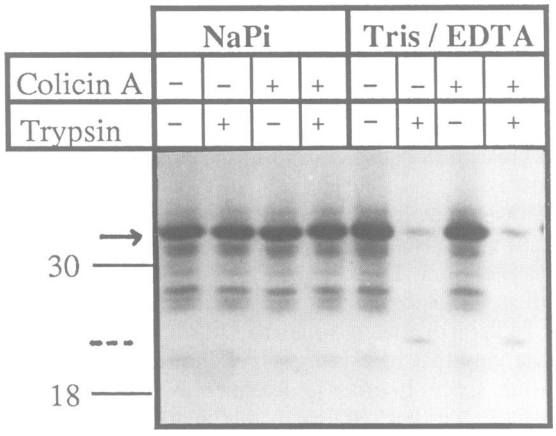

Fig. 7. Trypsin accessibility of OmpA: the experimental conditions are described in Material and methods. + and − respectively indicate the presence or absence of trypsin and colicin A in the cell suspension. The arrow shows the mature form of OmpA and (---) the typical degradation fragment of OmpA when trypsin has access to the periplasmic space. NaP_i (100 mM sodium phosphate, pH 6.8) and Tris−EDTA (10 mM Tris, pH 7.5, EDTA 0.5 mM) indicate the buffers in which the digestions were performed. Molecular mass markers are indicated on the left.

under which the pore had formed in the inner membrane) a major fragment of ~38 kDa was recovered with the cell envelope whereas the products of colicin A degradation found in the supernatant of these cells were fragments of

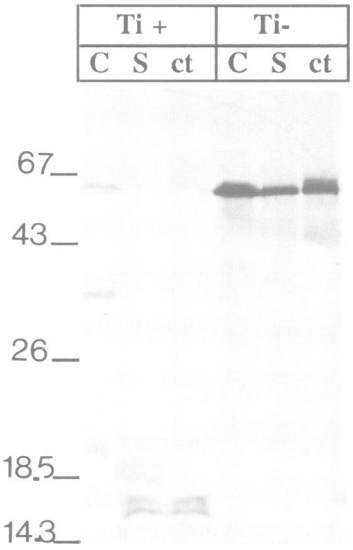

Fig. 8. Degradation pattern of ^{35}S-labelled colicin A after trypsin treatment: experiments were performed as described in Material and methods. C, S and ct correspond to the trichloracetic-precipitable material recovered in the cell pellet, the supernatant and to the colicin control in the absence of cells respectively. Ti^+ and Ti^- respectively indicate whether or not the cells and soluble colicin A were treated with trypsin. Molecular mass markers are indicated on the left.

15−18 kDa similar to those found for colicin A in solution. These results suggest that part of the colicin A molecule is protected from enzymatic cleavage when the pore is formed in the inner membrane.

Relationship between cell viability and the potassium content of colicin and trypsin-treated cells

Experiments similar to those described in Figure 6A were performed except that trypsin was added at different times (1, 1.5, 2, 4 and 5 min) after the efflux had been initiated by colicin A. K^+ efflux was arrested under all conditions and the cells retained 400, 350, 250, 100 and a few mM K^+ respectively. Aliquots of these cells were taken and their survival was determined as described in Material and methods. Figure 9 shows that the cell viability was strongly dependent on the time at which trypsin was added. Cells could be rescued when they retained high concentrations of K^+ but not any more when the internal concentration of K^+ was decreased below 250 mM. The same figure shows that cells which were not treated with trypsin could not be rescued from colicin action.

Discussion

The results presented in the first part of this paper suggest that a partial unfolding of the colicin A polypeptide may accelerate its translocation through the envelope of *E.coli*. These results were inferred from the lag time elapsing between the binding to the outer membrane of colicin A, either in a native or 'renatured' conformation, and the occurrence of potassium efflux in the inner membrane. In these experiments we assumed that the lag time constitutes a good approximation of the translocation time, providing that the number of colicin molecules added is high enough for the time required for binding to the receptor to be small in comparison with the translocation time. The time required

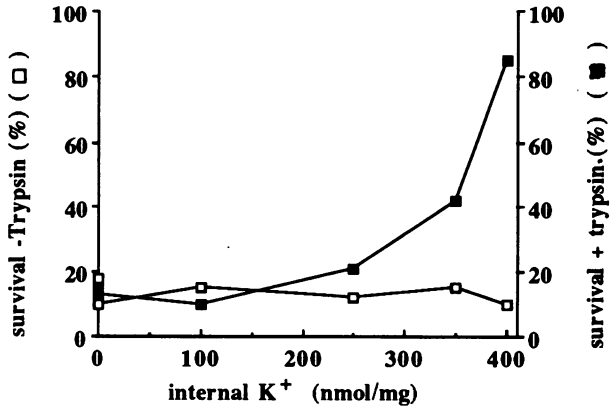

Fig. 9. Relationship between the potassium content of colicin-treated cells and viability. *Escherichia coli* cells were incubated in 'assay medium' in the presence of colicin A (multiplicity 30) as described in the legend to Figure 6A and trypsin was added at different times after the efflux had been initiated by colicin A (see text). Their potassium content was determined at the time where trypsin was added. Aliquots of these cell suspensions were taken for viability assay after addition of the trypsin inhibitor. Cells incubated with colicin A, treated (■) or not (□) with trypsin and taken for viability measurements 1, 1.5, 2, 4 and 5 min after colicin A addition respectively.

for translocation was reduced by at least a factor of two when colicin A was first caused to unfold in 8 M urea and directly diluted 200-fold in the cell suspension. It should be pointed out that the time elapsing between the dilution of the urea-treated colicin A and the opening of the pore was sufficient for colicin A to be partly refolded. Indeed, the fluorescence data show that colicin A elicits an initial rapid refolding step with a $\tau_{1/2} < 1$ min. Nevertheless, this 'intermediate folded state' (Creighton, 1990), which may be necessary for colicin A to recognize its receptor, suffices to accelerate its translocation. The further increase in the lag time depended on the time during which the colicin was renatured before being added to the cells, and a renaturation time of ~60 min was required for the lag time of the native colicin A to be recovered. This correlates well with the existence of a slow renaturation step, which was shown by fluorescence data to occur, and which probably corresponds to the recovery of interdomain interactions. The changes in the lag time were not accompanied by any change in the pore characteristics (rate of efflux, membrane potential threshold for closing) or in the number of pores formed in the inner membrane; this rules out the possibility that the decrease in the lag time may have resulted from an increase in the number of active colicin A molecules after urea treatment (i.e. that some aggregated colicin A may become active upon urea treatment). The decrease in the lag time therefore seems to be specifically related to the unfolding of colicin A and the rate-limiting step in the translocation may thus be the partial unfolding of the polypeptide chain.

Trypsin accessibility experiments strongly suggest that colicin A is still in contact with the external medium when forming the pore in the inner membrane. Indeed, addition of trypsin to the external medium, under conditions where the protease has access neither to the periplasmic space nor to the cytoplasmic membrane, sufficed to induce an immediate arrest of the K^+ efflux. This long range effect of trypsin was not due to a proteolytic cleavage of the receptor or of one of the proteins involved in the translocation of the colicin A but rather to a direct cleavage of colicin A. The proteolytic fragments of colicin A recovered in the cell envelope were different from those of colicin A in solution; thus although sensitive to proteolysis, part of the colicin A molecule is protected in the cell envelope. However, the arrest of K^+ efflux suggests that the fragment remaining in the envelope is not sufficient to allow pore activity. Colicin B, which has neither the same receptor nor the same translocation machinery as colicin A (Roos *et al.*, 1989) was also sensitive to trypsin action suggesting that it may be a general property of the pore forming colicins. Although colicins are elongated molecules (Lazdunski *et al.*, 1988) they are not long enough to cross the whole envelope; it is thus likely that in order to be accessible to trypsin they should remain unfolded upon pore formation.

The results of the trypsin experiments also yield new insights into the so-called 'trypsin rescue state'. Former experiments led to the conclusion that the action of trypsin on the pore forming colicins K and Ib is divided in stage 1 and stage 2 depending on whether the cell viability can be restored or not by adding trypsin (Nomura and Nakamura, 1962; Levisohn *et al.*, 1968). These results were further confirmed by Dankert *et al.* (1980), who showed that addition of trypsin early enough after colicin E1 allows a partial restoration of active transport in cells in which this transport has been initially inhibited by colicin E1. These authors also conclude that colicin E1 may acquire first a *trans*-envelope conformation corresponding to an intermediate state in the penetration of colicin through the cell envelope (stage 1) and then an irreversible lethal state (stage 2) at which colicin is no longer accessible to trypsin. We rather think that stages 1 and 2 correspond to the same *trans*-envelope conformational state of colicin but that the ability of the cells to be rescued by trypsin is only dependent on their K^+ content at the time when trypsin is added. When this K^+ concentration is decreased below 250 mM, cell viability is lost; it is likely that this depletion of internal K^+ which is accompanied by a decrease of the respiratory activity (Letellier and Boulanger, 1986; Boulanger and Letellier, 1988 and to be published) by a depolarization of the inner membrane (Gould and Cramer, 1977; Bourdineaud *et al.*, 1990) and a decrease in the internal ATP level (Fields and Luria, 1969) is responsible for the irreversible damage caused to the cells. It has been reported that bacteria treated with colicin E2 can also be rescued by trypsin after the colicins have begun to exert their lethal activity in the cytoplasm (Nose and Mizuno, 1968; Beppu *et al.*, 1972). In view of all the above results obtained with colicin A it is thus tempting to conclude that cytoplasmic acting colicins also span the envelope while exerting their lethal activity.

Which factors may then trigger this unfolding? The translocation of colicin A relies on a complex bacterial machinery involving the BtuB and OmpF receptors and the Tol proteins (Lazdunski *et al.*, 1988). It has been previously demonstrated that the BtuB binding site is located in the central domain of the colicin A polypeptide chain (Baty *et al.*, 1988), whereas the OmpF binding site is located in the N-terminal domain (Fourel *et al.*, 1990; Bénédetti *et al.*, 1991a). It is thus tempting to suggest that these interactions of colicin A with both BtuB and OmpF may trigger the first step in the unfolding of colicin A in the external membrane. Further unfolding into the periplasmic space may be favoured by the interaction of colicin A with the TolA protein, a protein associated with the inner membrane which extends

in the periplasmic space (Levengood and Webster, 1989; Levengood *et al.*, 1991). Indeed, it has been recently shown that the N-terminal domain of colicin A interacts with the C-terminal domain of TolA *in vitro* (Bénédetti *et al.*, 1991b). This interaction may be relevant to the *in vivo* translocation mechanism.

It is puzzling to observe such long distance effects of trypsin on the pore forming colicins. At present, we do not know which part of the colicin molecule is cleaved by trypsin and remains accessible to the external medium. This cleavage may either induce modifications of interdomain interactions in the unfolded colicins or disturb the interactions between the unfolded colicins and their translocation machinery resulting in closure of the colicin pores.

Many features of the translocation mechanism remain to be elucidated. Import into the inner mitochondrial membrane of most precursors appears to occur at contact sites between the two membranes (Schleyer and Neupert, 1985). Contact sites have been identified in *E.coli* cells (Ishidate *et al.*, 1986); the presence at these contact sites of proteins involved in the translocation of phage DNA has been recently demonstrated (Guihard *et al.*, 1992). The fact that colicin A spans the whole envelope upon pore formation suggests that the translocation of these toxins may also occur at these specific sites in the envelope. This hypothesis is now being investigated.

Materials and methods

Growth of bacteria

Escherichia coli cells from the GM1 strain [*ara* Δ(*lac pro*) thi (F′ *lac pro*)] (Sun and Webster, 1986) were grown at 37°C to an A_{650} of 0.5 (5×10^8 cells/ml) in minimum medium M9, supplemented with 0.1% casamino acids, thiamine (100 μg/ml) and 0.4% glucose (Meury *et al.*, 1985). The cells were washed and resuspended in 100 mM sodium phosphate buffer (pH 6.8) and kept on ice at a density of 5×10^{10} cells/ml. They were used within the 2 h following preparation.

Preparation of colicins A and B

Colicin A was prepared and purified from *Citrobacter freundii* strain CA 31 as previously described (Cavard and Lazdunski, 1979). For the preparation of ^{35}S-labelled colicin A, bacteria were grown in minimum medium M9 supplemented with thiamine (100 μg/ml), methionine assay medium (Difco Laboratories) and glycerol (0.4%). Cultures (200 ml) having an A_{650} of 1 were induced with mitomycin; after 60 min of induction [^{35}S]methionine (78 MBq) was added. After 4 h of induction, the culture was centrifuged and the ^{35}S-labelled colicin A was purified from the supernatant as described (Cavard and Lazdunski, 1979). The specific activity of the purified colicin A was 5 kBq/μg).

Colicin B (Schramm *et al.*, 1987) was prepared from *E.coli* C600 pcolB9. Colicin B was purified on a Pharmacia Mono Q ion exchange column (V.Geli, personal communication). The multiplicity is defined as the number of colicin molecules per cell.

Viability assays of cells treated with colicin A and trypsin

Aliquots of the cell suspensions (A_{650} = 1) used for K^+ efflux experiments were taken at different times, diluted 20-fold in LB medium, and the absorbance of the culture at 650 nm was followed as a function of time. The percentage of surviving cells was estimated from the A_{650} ratios of the colicin-treated and control cultures.

Measurement of $\Delta\Psi$ in cells treated with trypsin and in untreated cells

Escherichia coli cells were treated with EDTA–Tris in order to permeabilize the membrane to the potential cationic probe tetraphenyl phosphonium (TPP^+) as described previously (Bourdineaud *et al.*, 1990). For the measurement of $\Delta\Psi$ in trypsin-treated cells, the following procedure was used: the cells were first incubated with trypsin (250 μg/ml) for 10 min and trypsin inhibitor was then added (125 μg/ml). Cells were washed twice in the buffer before being treated with EDTA–Tris.

Determination of the K^+ content of the cells

The variations in the K^+ content of the cells (K^+_{in}) were determined by measuring the changes in the K^+ concentration in the external medium (K^+_{out}) with a K^+–valinomycin selective electrode (Boulanger and Letellier, 1988). The efficiency of the K^+–valinomycin electrode device was enhanced by modifying the reference electrode so as to increase both the stability of the signal (±5 mM internal K^+ resolution) and its time resolution (2 s). To estimate the total K^+ content of the bacteria, the cation was released from the cells by a cold osmotic downshock (Bourdineaud *et al.*, 1990). The amount of cation released was then estimated with the K^+ electrode. The voltmeter was connected to a computing device with which K^+_{in} can be directly determined from the value of K^+_{out}. K^+_{in} was expressed in nmol/mg cell dry weight (nmol/mg) assuming that 2×10^9 cells correspond to 1 mg cell dry weight. The 'assay medium' contained sodium phosphate buffer 100 mM, pH 6.8, 0.5 mM KCl and 0.4% glucose. The temperature was set at 37°C.

Analysis of the proteolytic fragments of urea-treated colicin A

Samples containing 1.4 μg of colicin A were incubated at room temperature for 15 min in a sodium phosphate buffer 100 mM pH 6.8 containing 8 M urea. Colicin A was then diluted 40-fold in the same buffer without urea. Aliquots were taken at various times and digested for 5 min at room temperature with 4 μg of thermolysin. Proteolysis was stopped by adding trichloracetic acid (12%) and the precipitate was layered at the top of a 12.5% SDS–PAGE. After blotting, the nitrocellulose was immunodetected as previously described (Granger-Schnarr *et al.*, 1988) using monoclonal anti-colicin A antibodies (3D6) (Cavard *et al.*, 1988) and secondary anti-mouse antibodies coupled to alkaline phosphatase.

Analysis of the proteolytic fragments of [^{35}S] colicin A after trypsin treatment

Escherichia coli cells were incubated at 37°C in 'assay medium' with ^{35}S-labelled colicin A (multiplicity 300) as described for the K^+ efflux experiments. Trypsin (250 μg/ml) and trypsin inhibitor (120 μg/ml) were added 1.5 min and 2.5 min after colicin A respectively. Cells were then centrifuged, the pellet and the supernatant were precipitated with trichloroacetic acid and aliquots layered on top of a 12.5% SDS–PAGE. The gels were fixed, dried and subjected to fluorography.

Trypsin accessibility experiments

Escherichia coli cells (10^9 cells, 0.5 ml) pretreated with colicin A (multiplicity 75) and untreated cells were incubated for 30 min either in the same assay medium as for the potassium efflux experiments or in Tris–HCl 10 mM, pH 7.5, EDTA 0.5 mM; buffers containing trypsin (50 μg/ml) or without trypsin were used. The cells were then washed twice and resuspended in the same buffer containing the soybean trypsin inhibitor (20 μg/ml). The cell envelopes were isolated after several freezing runs and loaded on a 12.5% SDS–PAGE. OmpA was immunodetected with polyclonal antibodies as described above.

Fluorescence experiments

Fluorescence measurements were carried out with a Kontron SFM 25 fluorimeter. To monitor the denaturation, colicin A was incubated with variable concentrations of urea for 30 min at 22°C and the tryptophan emission spectra were recorded at a λ ext = 280 nm. To determine the kinetics of renaturation, colicin A was incubated for 30 min in 8 M urea and then diluted 200-fold in 150 mM NaCl; aliquots were taken at different times and the tryptophan fluorescence emission was recorded (λ ext = 280 nm; λ em = 355 nm).

Circular dichroism spectra

These were performed with a Jobin Yvon autodichrograph in 1 mm thick cells. Colicin A (final concentration, 37 μg/ml) was diluted either in sodium phosphate buffer 100 mM, pH 6.8 or in 8 M urea. The circular dichroism spectrum of 'renatured' colicin A was performed 60 min after a 100-fold dilution of the urea-treated colicin in the buffer.

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