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## Unfolding of Colicin A during Its Translocation through the *Escherichia coli* Envelope as Demonstrated by Disulfide Bond Engineering\*

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Three double cysteine mutants, each possessing a disulfide bond in its pore-forming domain, were used to study the translocation of colicin A through the *Escherichia coli* envelope. These mutated colicins were able to exert their *in vivo* channel activity only after their disulfide bonds had been reduced by dithiothreitol. In solution, the reduction of the disulfide bonds by dithiothreitol was a slow process whose kinetics depended on the position of the disulfide bond ( $t_{1/2}$  varying between 35 and 100 s). This  $t_{1/2}$  was strongly decreased ( $t_{1/2} = 8-9$  s) upon prenaturation of the mutated colicins with urea. The  $t_{1/2}$  values of reduction of the mutants bound to *E. coli*-sensitive cells were similar to those of prenaturred colicins. This suggested that the interaction of the oxidized double cysteine mutants with the *E. coli* envelope triggered their unfolding. The disulfide bonds did not prevent but delayed the translocation of the colicins. The amplitude of the delay and the time at which it occurred during translocation depended on the position of the disulfide bond. We could discriminate between the delays accumulated during binding to the receptor and those during the translocation via OmpF and the Tol proteins.

To be translocated through biological membranes, polypeptides are usually synthesized with a targeting signal sequence (Blobel and Dobberstein, 1975), which interacts with cytosolic and membrane factors (Walter *et al.*, 1984). Cytosolic factors may not only select proteins destined for translocation but also maintain or generate a translocation-competent conformation (Collier *et al.*, 1988; Bochkareva *et al.*, 1988). Such a competent state is characterized by the loss of a stable tertiary structure (Randall and Hardy, 1986; Eilers *et al.*, 1988). In agreement with these requirements, it has been shown that denaturation of some precursor proteins with urea accelerates their translocation across membranes (Eilers *et al.*, 1988), whereas other precursors that possess disulfide bridges are not competent for translocation (Maher and Singer, 1986; Muller and Zimmermann, 1988; Jascur *et al.*, 1992). However, this does not appear to be a general rule. Indeed, Tani *et al.* (1990) first provided evidence that a non-linear polypeptide chain possessing disulfide bridges can be translocated through the secretory machinery of energized *Escherichia coli* membrane vesicles. These

data confirmed the model of a sequential translocation process of successive folded subdomains, as proposed by Maher and Singer (1986). Moreover, Pugsley (1992) has recently shown that pullulanase, a secreted protein possessing a disulfide bond, was translocated across the *E. coli* outer membrane.

Surprisingly, little is known about the import of macromolecules into the bacterial envelope. Pore-forming colicins are particularly suitable to study this import mechanism. These toxins first bind to a receptor located in the outer membrane and are then translocated through the envelope to reach their inner membrane target (Baty *et al.*, 1988). In the case of colicin A, translocation depends on the bacterial proteins TolA, TolB, TolQ, and TolR (Davies and Reeves, 1975). These proteins possess domains belonging both to the inner membrane and to the periplasmic space (Webster, 1991; Braun and Herman, 1993; Kampfenkel and Braun, 1993). It has recently been shown that translocation of colicin A takes place at contact sites between the inner membrane and the outer membrane and that colicin A remains in close contact with its machinery of translocation upon formation of the pore (Guihard *et al.*, 1994). Furthermore, colicin A is still accessible to the external medium after forming its pore in the inner membrane, and its pretreatment with urea accelerates its translocation through the envelope. It was concluded that colicin A unfolds during translocation and remains unfolded after forming the pore (Benedetti *et al.*, 1992).

The three-dimensional structure of the C-terminal pore-forming domain of colicin A consists of a bundle of 10  $\alpha$ -helices organized in three layers; helices 8 and 9 form a hydrophobic (helical) hairpin buried within the polypeptide (Parker *et al.*, 1989). Intramolecular disulfide bonds were introduced in this C-terminal domain. Three double cysteine mutants were studied, whose disulfide bridges connected helix 1 with helix 9, helix 5 with helix 6, and helix 9 with helix 10, respectively. The mutants in their oxidized form did not kill *E. coli* cells. Furthermore, the disulfide bond that connected helix 1 with helix 9 prevented the potential-independent insertion of colicin A in membrane vesicles, whereas the disulfide bridges that connected helix 5 with helix 6 or helix 9 with helix 10 prevented the potential-dependent channel opening in planar lipid bilayers (Duché *et al.*, 1994).

In this work, we have determined the kinetics of translocation of the oxidized mutants and their *in vivo* channel activities once reduced. These parameters were assessed from the measurement of the rate of efflux of cytoplasmic potassium induced by colicins in *E. coli* cells and from the lag time preceding this efflux (Bourdineaud *et al.*, 1990). We show that the disulfide bonds that cross-linked and rendered neighboring helices of the colicin A C-terminal domain non-dissociable do not prevent but slow down the rate of translocation of the toxin. Furthermore, we show that the delay occurs at different steps of the translocation and depends on the position of the disulfide bond. Our

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data also strongly suggest that the binding of colicin A to its receptor triggers its unfolding.

#### MATERIALS AND METHODS

**Bacterial Strain and Media**—*E. coli* cells from GM1 strain (*ara* Δ (*lac pro*) *thi* (*F' lac pro*)) (Sun and Webster, 1986) were grown at 37 °C to an  $A_{650}$  of  $0.5 (5 \times 10^8 \text{ cells/ml})$  in 2.3BT medium (5 g of Bacto-tryptone, 8 g of nutrient broth, 5 g/liter NaCl, 10 mM Tris-HCl (pH 7.2), 1 mM  $\text{CaCl}_2$ ). The cells were washed and resuspended in 100 mM sodium phosphate buffer (pH 7.2) and kept on ice at a density of  $5 \times 10^{10}$  cells/ml. They were used within 2 h after preparation.

**Site-directed Mutagenesis**—The double cysteine mutants I26C/G176C (H1/H9), A91C/E115C (H5/H6), and G184C/F191C (H9/H10) and the corresponding single cysteine mutants I26C, G176C, A91C, E115C, G184C, and F191C were obtained by site-directed mutagenesis as described previously (Duché *et al.*, 1994).

**Protein Purification**—Colicin A and the double cysteine mutants were prepared and purified from *E. coli* W3110 strain as described previously (Duché *et al.*, 1994). Colicins were solubilized in 50 mM Tris-HCl (pH 8.0), and 0.01% Triton X-100 was added to prevent oligomerization. Disulfide bond formation of the purified mutated colicins was titrated as described previously (Duché *et al.*, 1994).

**Measurement of Colicin-induced  $K^+$  Efflux**—The variations in the cytoplasmic  $K^+$  content of the cells induced by colicins were determined by measuring the changes of the  $K^+$  concentration in the external medium with a  $K^+$ /valinomycin-selective electrode as described previously (Boulanger and Letellier, 1988). Cells ( $2 \times 10^9/\text{ml}$ ) were incubated 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. Under these conditions cells accumulated  $K^+$  via the TrKA system to a steady state level of 400–450 nmol/mg of cell, dry weight (Boulanger and Letellier, 1988). Colicins were added at given multiplicities (number of colicin molecules/bacterium), and the changes in the  $K^+$  concentration were determined as a function of time. The initial rate of  $K^+$  efflux was determined in the linear part of the  $K^+$  efflux curve as described previously (Bourdineaud *et al.*, 1990).

**Reduction of the Double Cysteine Mutants and Determination of *In Vivo* Channel Activity of the Mutated Colicins**—Colicins were reduced by DTT<sup>1</sup> either in their native conformation or once denatured in 8 M urea. Mutated colicins and wild type colicin A (1 mg/ml) were incubated at 37 °C in 50 mM Tris-HCl (pH 8.0), 0.01% Triton X-100 with or without 8 M urea for given times and at given concentrations of DTT. The DTT solution was prepared extemporaneously in 50 mM Tris-HCl (pH 8.0) previously degassed and equilibrated with argon. For measurement of the *in vivo* channel activity, DTT (urea)-treated colicins were diluted in 50 mM Tris-HCl (pH 8.0) containing the same concentration of DTT as for the reduction and added to the cells at a multiplicity of 200. After dilution the final concentration of urea was 40 mM (under these conditions colicin A remains unfolded for at least a few minutes (Benedetti *et al.*, 1992)). The channel activity of the double cysteine mutants was determined by measuring the initial rate of  $K^+$  efflux and the lag time preceding this efflux as a function of colicin multiplicity (Bourdineaud *et al.*, 1990). These rates of efflux were compared with those obtained with wild type colicin A pretreated with DTT. Reduction of the disulfide bonds was considered to be total when, for a given multiplicity, the rate of  $K^+$  efflux of the mutant attained a value close to that of the wild type colicin. Neither DTT (5 mM) nor urea (40 mM) alone had any effect on the  $K^+$  content of *E. coli* cells or on the response of the  $K^+$ /valinomycin electrode.

#### RESULTS

**Kinetics of *In Vitro* Reduction of the Disulfide Bond of Native and Denatured Colicins**—The three double cysteine mutants that possess a disulfide bond connecting helix 1 with helix 9 (I26C/G176C), H1/H9, helix 5 with helix 6 (A91C/E115C), H5/H6, and helix 9 with helix 10 (G184C/F191C), H9/H10, respectively, did not induce any efflux of cytoplasmic  $K^+$  when added to *E. coli* cells. This suggested that the presence of the disulfide bonds prevents the formation of the channel in the inner membrane. When the mutated colicins were first incubated with 5 mM DTT and then added to *E. coli* cells, they regained channel activity (Duché *et al.*, 1994). Once reduced, the double cysteine mutants behaved like wild type colicins; the initial rate of  $K^+$

TABLE I  
Initial rate of  $K^+$  efflux and lag time preceding this efflux for the mutants reduced either *in vitro* or *in vivo* and for the colicin A wild type

Mutant	<i>In vitro</i> reduction		<i>In vivo</i> reduction, initial rate of $K^+$ efflux nmol/min-mg
	Initial rate of $K^+$ efflux	Lag time	
	nmol/min-mg	s	
H1/H9	280	33	290
H5/H6	440	27	430
H9/H10	330	35	310
WT	450	27	440

efflux increased with increasing multiplicity and saturated at a multiplicity of 200 (data not shown). The initial rate of  $K^+$  efflux of the reduced H5/H6 mutant was identical to that of the wild type colicin A, whereas the rate of  $K^+$  efflux of the two other reduced mutants was slightly lower (Table I).

To determine the kinetics of *in vitro* reduction of their disulfide bonds, mutated colicins were incubated for variable times with increasing concentrations of DTT and then added to *E. coli* cells. The channel activity of the DTT-treated colicins was then determined by measuring the initial rate of  $K^+$  efflux and by comparing this rate with that obtained with wild type colicin A (see "Materials and Methods"). The concentrations of DTT and the times needed to regain half of the channel activity of the different mutants ( $\text{DTT}_{1/2}$  and  $t_{1/2}$ , respectively) are given in Table II (top, a, and bottom, a). Full reduction of mutants H9/H10 and H1/H9 required 5 mM DTT whereas reduction of mutant H5/H6 only required 2 mM DTT. The time needed for full reduction of the mutants treated with 5 mM DTT also depended on the position of the disulfide bond; it was 4-fold shorter for the H5/H6 mutant ( $t = 120$  s) than for the two other mutants ( $t = 450$  and  $500$  s for mutants H1/H9 and H9/H10, respectively). This suggests that the disulfide bond that connected helix 5 with helix 6 is probably more accessible to DTT than the disulfide bonds connecting helix 1 with helix 9 or helix 9 with helix 10. Predenaturation of the colicin A mutants in urea before DTT treatment substantially reduced the concentration of DTT ( $\text{DTT}_{1/2} \approx 0.1$  mM) and the time ( $t_{1/2} = 8$ –9 s) needed for reduction of their disulfide bonds (Table II (top, b and bottom, b)). These results are consistent with a better accessibility of the disulfide bonds in the unfolded colicins.

**Disulfide Bonds in the Pore-forming Domains of the Mutated Colicins neither Prevent Their Unfolding nor Their Translocation**—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 (Bourdineaud *et al.*, 1990). At a multiplicity of 200 and at 37 °C, the lag time equaled 27 s for wild type colicin A and 27, 33, and 35 s, respectively, for the H5/H6, H1/H9, and H9/H10 mutants whose disulfide bonds were reduced before their addition to the cells. These results indicate that the introduction of the mutations independently of the disulfide bond is sufficient to delay the translocation by 6–8 s. Interestingly, the single mutation H10 (F191C) slowed down the translocation by 6 s, but the single mutations H1 (I26C) or H9 (G176C) caused no delay although the corresponding double cysteine mutant was delayed (data not shown).

<sup>1</sup> The abbreviation used is: DTT, dithiothreitol.

TABLE II

The disulfide bonds of the mutated colicins become more accessible to DTT when the mutants undergo *in vitro* denaturation or are translocated through the *E. coli* envelope

Top, a and b, and bottom, a and b, the double cysteine mutants H1/H9, H5/H6, and H9/H10 were incubated at 37 °C in 50 mM Tris-HCl (pH 8.0) either with various concentrations of DTT for 10 min (top) or with 5 mM DTT for various times (bottom). The DTT-treated mutants were either in their native forms (a) or denatured in 8 M urea (b). They were then added to *E. coli* cells at a multiplicity of 200. Their channel activities were inferred from the measurement of K<sup>+</sup> efflux as described under "Materials and Methods." Top and bottom show the concentration of DTT and the time of incubation in 5 mM DTT needed to reduce half of the disulfide bonds, respectively. Top and bottom, c, mutated colicins were preincubated for 1 min with *E. coli* cells at 37 °C and at a multiplicity of 200. Then various concentrations of DTT were added, and channel activity was determined as described under "Materials and Methods." Top, c, concentration of DTT required to reduce half of the disulfide bonds. Bottom, c, shows the time elapsing between addition of 5 mM DTT and the onset of a K<sup>+</sup> efflux. Values are the mean of three different experiments.

Mutant	DTT <sub>1/2</sub> (mM)		
	a	b	c
H1/H9	2.1 ± 0.1	0.09 ± 0.02	0.8 ± 0.09
H5/H6	0.6 ± 0.08	0.08 ± 0.02	0.6 ± 0.1
H9/H10	2 ± 0.15	0.13 ± 0.05	1.15 ± 0.15

	t <sub>1/2</sub> of disulfide bond reduction (s)		
	a	b	c <sup>a</sup>
H1/H9	85 ± 5	9 ± 1	7 ± 1
H5/H6	35 ± 5	8 ± 1	8 ± 1
H9/H10	100 ± 10	9 ± 1	10 ± 1

<sup>a</sup> Time needed for *in vivo* reduction (s).

To determine if the the disulfide bonds could be reduced *in vivo*, oxidized colicins were incubated with the cells at a multiplicity of 200 for 1 min at 37 °C (*i.e.* a time longer than that needed for full translocation of the reduced colicins, see above), and then DTT was added at various concentrations and the rate of efflux was determined. Whatever the mutant was, addition of 5 mM DTT resulted in a K<sup>+</sup> efflux whose initial rate was identical to that of the colicin mutants reduced *in vitro* (Table I). DTT alone had no effect on the wild type colicin A activity (not shown). This K<sup>+</sup> efflux started 7–10 s after DTT addition (Table II, bottom, c). This lag is shorter than the time needed for full translocation (27–35 s). This suggests that the oxidized colicins are translocated at least partially, despite their disulfide bond, and that the disulfide bond can be reduced once the colicins are inserted into the envelope. Increasing the time of incubation of the cells with the oxidized colicins from 1 to 5 min did not modify the time (7–10 s) elapsing between DTT addition and K<sup>+</sup> efflux (not shown). Thus 7–10 s may represent the maximum time needed for *in vivo* DTT action. This value is 60-fold smaller than the time needed for *in vitro* reduction of the native mutants and similar to the t<sub>1/2</sub> needed for *in vitro* reduction of the colicins denatured by 8 M urea (Table II, bottom). Accessibility of the disulfide bond *in vivo* resembles that of *in vitro* denatured colicins. Thus, oxidized colicins probably unfolded once they interact with the bacterial envelope. Unfolding is therefore taking place independently of the presence of the disulfide bonds.

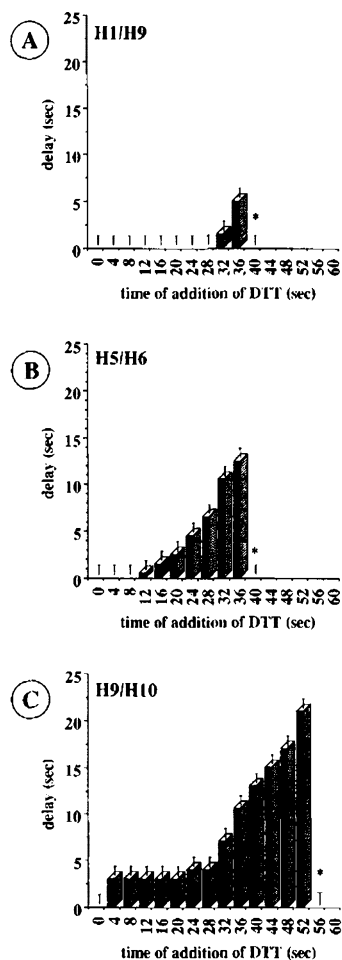
Interestingly the concentration of DTT needed for *in vivo* reduction of the H1/H9 and H9/H10 mutants was slightly smaller than the concentration needed for their *in vitro* reduction (Table II, top). This is in agreement with a better accessibility of the disulfide bonds *in vivo* than *in vitro*.

**The Presence of Disulfide Bonds in the Pore-forming Domain Slows Down the Translocation of the Mutated Colicins**—The preceding experiments indicate that the presence of disulfide bonds does not prevent translocation of the mutated colicins.

The disulfide bonds may, however, affect the kinetics of this translocation. To follow this kinetics, oxidized colicins were incubated with bacteria for times ranging between 4 and 60 s. The disulfide bonds were then reduced by DTT (5 mM), and the time elapsing between DTT addition and K<sup>+</sup> efflux on the one hand and the initial rate of this efflux on the other hand were determined. Whatever time of addition of DTT was, the initial rate of K<sup>+</sup> efflux was identical to that measured for the mutants reduced *in vitro* (data not shown). Since this initial rate is related to the number of channels formed (Bourdineaud *et al.*, 1990), this indicates that the mutated colicins are translocated with the same efficiency whatever the time at which the disulfide bonds were reduced. The translocation time, however, strongly depended on the position of the disulfide bonds and on the time at which they were reduced. A delay was calculated that is defined as the difference between the lag time determined for the mutant reduced *in vivo* and the lag time of the mutant reduced *in vitro* (see Table I). These delays were corrected to take in account the time needed for *in vivo* reduction of the disulfide bond by DTT; we assumed that disulfide bonds were reduced in 7–10 s whatever the time at which DTT was added. These 7–10 s were therefore subtracted from each value of the delay. Fig. 1 represents the corrected delays as a function of time of addition of DTT. Full translocation of the oxidized mutants was achieved in 38 s (± 2 s) for the H1/H9 and H5/H6 mutants and 54 s (± 2 s) for the H1/H9 mutant since starting from these values efflux of K<sup>+</sup> always took place 7–10 s after DTT addition. Given the lag time of the mutants reduced *in vitro* (33, 27, and 35 s for the H1/H9, H5/H6, and H9/H10 mutants, respectively), this suggests that the presence of the disulfide bonds slows down the translocation by 5, 11, and 19 s, respectively.

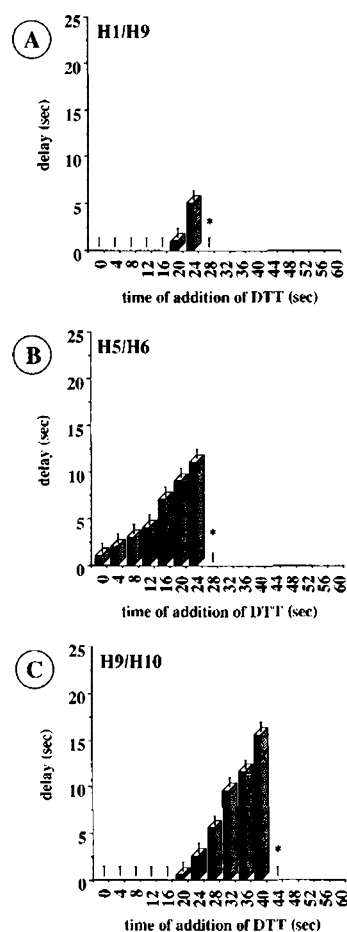
These delays were not accumulated at all stages of the translocation. The disulfide bond connecting helices 1–9 perturbed the translocation only slightly since there was no detectable delay for the first 32 s and since the overall translocation was only delayed by 5 s (Fig. 1A). The disulfide bond connecting helix 5 with helix 6 affected the translocation at an early stage since the delay started when DTT was added 12 s after mutant H5/H6 (Fig. 1B). Finally, a constant delay of 4 s was observed during 20 s for the mutant H9/H10, and then this delay increased to finally reach 19 s. Therefore, the presence of a disulfide bond connecting helix 9 with helix 10 has a strong effect on the translocation (Fig. 1C). Interestingly, the corrected delay was equal to 0 if reduction of the disulfide bonds occurred during the first seconds after the addition of the mutants (Fig. 1). In other words, under these conditions, reduction of the disulfide bond occurred in 7–10 s (see preceding paragraph). If the oxidized colicins were bound to the envelope in their native form, we would have expected the reduction to require the same time as *in vitro* (*i.e.* 120–450 s depending on the mutant (see preceding paragraph)) and therefore the K<sup>+</sup> efflux to occur 120–450 s after DTT addition. Since the time needed for reduction was equal to that determined for *in vitro* reduction of denatured colicins (see Table II, bottom, c), this suggests that, as soon as the colicins are in contact with the outer membrane surface they are, at least partially, unfolded.

**The Translocation Time Is Significantly Reduced by Bypassing the BtuB Receptor**—The complex behavior of the mutants suggests that the translocation is a multistep process. Translocation of colicin A requires the receptor BtuB, the porin OmpF, and the Tol machinery (Benedetti *et al.*, 1989). Under "by-pass" conditions (*i.e.* when cells and colicins are incubated at low ionic strength) colicin A directly binds to OmpF (Benedetti *et al.*, 1989). Under these conditions, the lag time of wild type colicin A is decreased from 27 to 18 s (Guihard *et al.*,



**FIG. 1. The disulfide bonds delay the translocation of the double cysteine mutants.** Cells ( $2 \times 10^9$ /ml) were incubated at  $37^\circ\text{C}$  in sodium phosphate buffer (100 mM, pH 7.2) containing 0.2% glucose and 0.5 mM KCl. The double cysteine mutants were added to the cells at time 0 and at a multiplicity (number of colicins/bacterium) of 200; after incubation for times ranging between 4 and 60 s the disulfide bonds were reduced by the addition of 5 mM DTT. The time elapsing between colicin addition and  $\text{K}^+$  efflux (lag time) was measured. A delay was calculated, which is defined as the difference between the lag time determined for the mutant reduced *in vivo* and the lag time of the mutant reduced *in vitro*. 7–10 s were subtracted from the found values to take in account the time needed for the disulfide bond to be reduced by DTT (see Table II, bottom, c and text). A, B, and C represent the corrected delays calculated for mutants H1/H9, H5/H6, and H9/H10 as a function of the time of addition of DTT. \*, starting from this time of addition of DTT, the corrected delays were equal to 0. The error bars correspond to the mean of three experiments.

1994). By-passing the BtuB receptor also shortened the lag time of the double cysteine mutants reduced *in vitro*: 21, 16, and 28 s for mutants H1/H9, H5/H6, and H9/H10, respectively (data not shown) compared with 33, 27, and 35 s at high ionic strength. The oxidized mutated colicins were also reduced at different times after they were added to *E. coli* cells. Fig. 2 shows the corrected delays introduced by the disulfide bonds as a function of the time of addition of DTT. The “by-pass” of the BtuB receptor shortened the total translocation time. However, this shortening affected especially the outset of the translocation and not the second part, which was very similar to that observed at high ionic strength. For the double cysteine mutant H1/H9, the delay started after 20 s (Fig. 2A) as compared with 32 s at high ionic strength (Fig. 1A). However, under both high and low ionic strengths, translocation of the oxidized mutant was only delayed by 8 s as compared with the reduced one. The differences in the delays between “by-pass” and “normal” con-



**FIG. 2. Delay in the translocation of the mutants under by-pass conditions.** Experiments were performed as described in the legend of Fig. 1, except that the assay medium contained 10 mM sodium phosphate instead of 100 mM sodium phosphate.

ditions were even more striking in the case of the mutants H5/H6 and H9/H10. For the mutant H9/H10, the constant delay of 4 s observed at high ionic strength during 20 s totally disappeared, and only a delay starting after 20 s and ending after 44 s was observed. This was also the case for the mutant H5/H6 for which the initial step lasting 12 s (in which no delay was observed) disappeared.

#### DISCUSSION

In this work we have used three colicin A mutants to study the kinetics of translocation of this pore-forming toxin through the *E. coli* envelope and its state of folding during translocation. Each of these mutants possesses a disulfide bond connecting two  $\alpha$ -helices of its pore-forming domain. The oxidized colicins are inactive, but as DTT was added *in vitro*, they recovered full channel activity as judged from their capacity to induce an efflux of cytoplasmic  $\text{K}^+$  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. Several arguments support the proposal that *in vivo* reduction is due to DTT and not to catalysis by a protein possessing a disulfide oxidoreductase activity. First, the mutated colicins never recovered channel activity without DTT treatment. Second, the recovery of channel activity depended on DTT concentration the same way whether reduction was performed *in vitro* or *in vivo* (data not shown). Third, disulfide bonds were reduced as efficiently whether the colicins were bound to the surface of the outer membrane or close to the inner membrane. These arguments rule out the possibility that a unique enzyme

would catalyze this reduction. DsbA is a protein localized in the periplasm of *E. coli*, and it is involved in the formation of disulfide bonds of exported proteins (Bardwell *et al.*, 1991, 1993). The involvement of DsbA was also ruled out since *in vivo* reduction needed the same time in a wild type strain and in a *dsbA* mutant (data not shown). DsbB is already in a reduced form, and it was hypothesized to specifically oxidize DsbA; therefore the involvement of DsbB was also ruled out (Bardwell *et al.*, 1993).

Interestingly, disulfide bonds were reduced faster *in vivo* than *in vitro*, as clearly demonstrated in two experiments. First, oxidized colicins were incubated with bacteria for a time longer than that needed for full translocation and they were then reduced. Under these conditions the efflux of K<sup>+</sup> started 7–10 s after DTT addition; second, DTT was added immediately after the oxidized colicins had bound to the cell envelope. Again full translocation required only 7 s more than translocation of the colicin reduced *in vitro*. These 7–10 s may thus represent the time needed for disulfide bonds to be reduced *in vivo*. *In vitro* the  $t_{1/2}$  of reduction varied between 85 and 100 s. This  $t_{1/2}$  was, however, only 8 s if the colicins were first denatured with urea before DTT was added. Because the value found *in vivo* was very close to that found for the colicins denatured *in vitro*, it suggested that the *in vivo* accessibility of the disulfide bonds to DTT resembled that of denatured colicins. It is therefore likely that the oxidized colicins undergo a conformational change once they are in the envelope and they are at least partially unfolded. Furthermore, the second experiment indicates that this unfolding takes place during the first seconds following binding of the oxidized colicins to the *E. coli* envelope. The first experiment allows us to conclude that the presence of intramolecular disulfide bonds does not prevent the translocation and that the pore-forming domain of the oxidized colicins is close to the inner membrane since efflux of K<sup>+</sup> occurred immediately after the disulfide bonds had been reduced. The fact that colicin A is translocated across the outer membrane despite the presence of disulfide bonds is in agreement with what Pugsley (1992) reported; a disulfide bond must be formed for pullulanase to be translocated through the outer membrane and secreted into the external medium. Hirst and Holmgren (1987) also demonstrated that the subunits of the heat-labile enterotoxin produced by *Vibrio cholerae* assembled into oligomers within the periplasm before they were secreted into the external medium. However, the fact that polypeptides folded into a tertiary or even a quaternary conformation can be translocated does not appear to be a general rule since proteins possessing an intramolecular disulfide bond were not translocated across the membranes of mitochondria (Jascur *et al.*, 1992) or endoplasmic reticulum (Maher and Singer, 1986; Muller and Zimmermann, 1988).

The mutations introduced in the pore-forming domain, independently of the formation of the disulfide bond, were sufficient to slow down the rate of translocation of the colicins. Translocation of the reduced double cysteine mutants H1/H9 and H9/H10 required 8 s more than that of wild type colicin A. Interestingly, the single mutation in H10 slowed down the translocation by 6 s, but the single mutants in H1 or H9 were not delayed. It is worth noting that these mutants were also affected in their channel activities since the initial rate of K<sup>+</sup> efflux was significantly smaller for the H1/H9 and H9/H10 mutants (280 and 330 nmol/min·mg, respectively) than for the wild type colicin. The reduced mutant H5/H6 behaved differently since its rate of translocation and its channel activity were similar to those of wild type colicin A.

Disulfide bonds did not prevent translocation but slowed it down. The translocations of the oxidized mutants H1/H9, H5/

H6, and H9/H10 were delayed by 5, 11, and 19 s ( $\pm 2$  s), respectively, as compared with those of reduced colicins. It is interesting to note that the delay increased with decreasing the length of the loop connecting the 2 cysteine residues forming the disulfide bond: 149, 23, and 6 residues for H1/H9, H5/H6, and H9/H10, respectively. The translocation could be roughly divided into two stages. During the first stage the presence of the disulfide bond did not delay or only slightly delayed by 4 s (mutant H9/H10) the rate of translocation. During the second stage, the three mutants behaved quite differently; the disulfide bonds in mutants H5/H6 and H9/H10 strongly delayed the translocation whereas the disulfide bond in H1/H9 slowed down only slightly the translocation. Interestingly, the translocation featured different kinetics when the receptor BtuB was by-passed and when colicin A bound directly on the porin OmpF; the first stage was strongly shortened and even became undetectable while the second stage remained unchanged. It is thus likely that the first stage corresponds to the binding of colicin A to BtuB and to the interaction of the resulting complex with OmpF, while the second stage corresponds to the translocation of colicin A through the envelope via the Tol proteins. Since for all the mutants full translocation lasted  $12 \pm 2$  s less than when BtuB was by-passed, this suggests that this value roughly represents the time needed by colicin A to bind to BtuB and for the BtuB-colicin complex to interact with OmpF. This is also the time during which unfolding of colicin A would take place. Previous experiments showed that colicin A spans the whole envelope and is therefore unfolded when forming its pore in the inner membrane (Benedetti *et al.*, 1992). The present data show that colicin A unfolds at an early stage of the translocation, *i.e.* upon interaction with its receptor. It is also likely that this change in conformation allows colicin A to interact with its translocation machinery and to form a complex with the Tol proteins, which would span the whole envelope (Guihard *et al.*, 1994).

One important feature of these results is that a modification in the pore-forming domain has a pronounced effect on the translocation. The polypeptide chain of colicin A is organized in three distinct domains, and a specific function has been attributed to each of these domains: the central domain is involved in receptor binding, the N-terminal domain is required for translocation through the envelope, and the C-terminal domain carries the pore-forming activity (Baty *et al.*, 1988). Our data suggest that these different domains are interdependent.

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