JOURNAL OF BACTERIOLOGY, Jan. 1982, p. 306-315
0021-9193/82/010306-10$02.00/0

Vol. 149, No. 1

Fragmentation of Colicins A and E1 by Cell Surface Proteases

ROBERT N. BREY

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received 11 June 1981/Accepted 12 August 1981

Interaction of either colicin A or E1 with the surface of *Escherichia coli* cells resulted in extensive cleavage of the colicins into many peptide fragments in the molecular weight range of 10,000 to 30,000 released into the supernatants of colicin-cell mixtures. The protease inhibitor *p*-aminobenzamidine inhibited the cleavage of colicin A and enhanced colicin killing activity, suggesting that proteolysis is not required for the killing action of colicin. Fragments derived from the supernatants of the mixtures were inactive against sensitive cells. Proteolytic activity against both colicins was localized primarily in the outer membrane fraction of the cell envelope. At least two distinct protease activities appear to be present. Examination of the patterns of cleavage and inactivation of the colicins by a series of resistant mutants indicates that specific colicin receptors play no essential role in colicin proteolysis. In addition, evidence is presented that adsorption of colicin to specific receptors is a reversible process.

The initial step in the killing of sensitive *Escherichia coli* cells by colicins involves a binding to specific receptors located in the bacterial outer membrane (12, 18, 20, 23, 29, 30). Events involved in cell killing subsequent to receptor binding appear to involve the participation of a number of proteins, in that mutational changes without loss of receptor function give rise to strains refractory to various colicins (tolerant mutants) and evince other membrane defects such as sensitivity to detergents or dyes (22).

At least with respect to colicins E2 and E3, the colicin molecule presumably crosses the cell wall barrier and the cytoplasmic membrane to act upon intracellular targets (24, 32). Colicin E1 may not need to penetrate the membranes since colicin molecules covalently coupled to Sepharose can kill sensitive cells (14). A part of the molecule, or the whole molecule, must span the outer membrane to produce channels for ions and small molecules in the inner membrane, resulting in a loss of membrane potential (11, 33, 38, 39, 41).

The mechanism whereby colicins are translocated from receptor sites to their specific sites of action is open to speculation. Some reports have proposed that proteolytic cleavage of colicins by interaction with cells or isolated membrane fractions is involved in cell killing. Cavard and Lazdunski (2) have shown that colicin E4, upon interaction with sensitive cells, is split into two peptide fragments, which are inactive against bacterial cells. These authors suggested that cleavage is an integral process in cell killing since resistant mutants failed to mediate colicin cleavage. In a more recent report, Bowles and Konisky showed that colicin Ia was cleaved by a protease that appeared to operate independently of receptor function (1).

Experiments reported in this study were undertaken to clarify what role, if any, fragmentation plays in the mechanism of killing by colicins. The findings indicate that proteolysis of colicins A and E1 does occur but is probably not involved in killing by these colicins. Specific receptors are apparently not required for cleavage of either colicin. At least two distinct colicin-degrading protease activities are associated with the *E. coli* outer membrane.

MATERIALS AND METHODS

Bacterial strains and media. For experiments on the cleavage or inactivation of colicin, cells were grown in L broth at 37°C to a density of 2×10^8 cells per ml before harvesting. For the experiment in Table 3, cells were grown in L broth lacking added NaCl, conditions under which synthesis of outer membrane protein 1a is not repressed (28, 31). For preparation of outer or inner membranes, 1 ml of an overnight culture in L broth was inoculated into 2 liters of Ozeki salts medium (22) containing 54 mM glycerol, 0.5% Casamino Acids, and 5 μg of thiamine HCl per ml. Cultures were grown at 37°C to a density of 10^9 cells per ml before harvesting. Genetic properties and colicin sensitivities of bacterial strains are described in Table 1.

Preparation of membranes. Outer membranes were prepared as the Triton X-100–insoluble fraction of membrane vesicles obtained by lysis of whole cells in a French pressure cell operating at 15,000 lb/in^2 by the methods described by Schnaitman (5, 34). Alternatively, the membrane fraction obtained by French pressure cell lysis was separated on sucrose density gradients by the method outlined by Osborn et al. (26).

TABLE 1. Bacterial strains

Strain designation	Genotype	Colicin resistance[a]	Source (reference)
C600	*thr leu lac tonA*		Luria collection
A586	*thr leu lac tonA tolC*	E1	(22)
A592	*thr leu lac tonA tolA*	E1, E2, E3, K, A	(22)
A593	*thr leu lac ton tolB*	E2, E3, K, A	(22)
A597	*thr leu lac tonA ompF*	A	(22)
A598	*thr leu lac tonA tsx*	K	Luria collection
RB314	*thr leu lac tonA btuB*	pE1, pA, E2, E3	Spontaneous BF23-resistant C600
RB330	*thr leu lac tonA btuB ompF*	pE1, A, E2, E3	Spontaneous BF23-resistant A597
JSM20	C600 *zig-2*::Tn*10 ampA*		J. Suit
CJ30	*trp*(Am) *lac*(Am)		C. Plate (27)
Colicinogenic strains	**Host strain**	**Plasmid**	
JSA 1	CJ30	pDMS630 colicin E1-producing	J. Suit
A881	W3110	colE3	Luria collection
A737	QR47	colE2	Luria collection
610	O6:H16, 23	colA	Luria collection
LK235	LK235	colA	Luria collection
NO33	K235	colK	M. Nomura

[a] Colicin sensitivity of each strain was determined by the halo test. Of the five colicins examined in this study, the ones listed are those to which a particular strain is refractory; p denotes partial or incomplete resistance. In formal terms, resistance can be defined as a phenotype which results from a deficiency in specific colicin receptors. Tolerant mutants are refractory to a particular colicin but are still able to bind the colicin since receptors are intact. A mutation to BF23 resistance (formerly termed *bfe*, but here called *btuB*) results in loss of colicin E receptors (6, 43). A mutation at *ompF* results in loss of functional colicin A receptors (this study). *ompF* mutants are missing outer membrane protein 1a (8).

Outer membranes were suspended to 5 to 10 mg of protein per ml in 0.01 M HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid), pH 7.4, and kept frozen at −70°C. Succinate dehydrogenase activity was measured as described by Osborn et al. (26), and 2-keto-3-deoxyheptonic acid as an index of outer membrane purity was measured by the method of Weissbach and Hurwitz (42).

Purification of colicins. Colicin A was purified as follows. An overnight culture of strain LK235 was diluted 1:50 into L broth (typically, 4 liters) and incubated with aeration at 37°C. When cell density reached 3×10^8 cells per ml, 1 μg of mitomycin C per ml was added to induce colicin synthesis, and cells were aerated for an additional 2.5 h. The induced culture was chilled and harvested, and the cell pellet was washed once in L broth, weighed, and transferred to a chilled mortar. Alumina was added at a ratio of 2.5 g to 1 g (wet weight) of cells, and the cell mass was ground until a paste stage was achieved. The paste was suspended in a minimum volume of 0.1 M potassium phosphate, pH 7.0, containing 1 M NaCl and was transferred to a centrifuge tube, to which DNase (10 μg/ml), RNase (10 μg/ml), and 5 mM $MgSO_4$ were added. After a 5-min incubation at room temperature, alumina was separated from the lysate by centrifugation at 3,000 × *g* for 10 min. The supernatant fluid was then centrifuged at 100,000 × *g* for 1 h to remove cell membranes and cell debris. Ammonium sulfate was added to the clear supernatant to 20% saturation. The precipitate from this step was discarded, and the supernatant fluid was treated with ammonium sulfate to 60% saturation. The resultant precipitate was dissolved in a minimum volume of 0.01 M sodium phosphate (pH 7.1) and dialyzed overnight against several liters of the same buffer. The dialyzed material was passed over a DEAE-Sephadex column pre-equilibrated with 10 mM sodium phosphate. The void-eluting material was retained and chromatographed on a Bio-Gel P-60 column with elution by 0.10 mM sodium phosphate, pH 7.1. Material eluting at the void volume of this column was pure colicin A. Colicin A was stored at −70°C in 50-μl portions at concentrations which ranged from 1 to 20 mg/ml. The specific activity of the final material varied from 2×10^{13} to 2×10^{14} killing units/mg of protein, which is equivalent to 1 cell-killing event per 100 or 1,000 molecules, with single-hit kinetics assumed.

Colicin E1 was purified from mitomycin C-induced strain JSA1 by the procedure of Schwartz and Helinski (35). The specific activity of the colicin was approximately 1.3×10^{15} killing units/mg, which corresponds to 1 active molecule in 10.

Killing activity and titration of colicins. Killing by colicins was measured by mixing suitable dilutions of colicins in 0.01 M sodium phosphate, pH 7.1, with approximately 1.5×10^8 cells per ml in L broth at 37°C. At times after the addition of colicin, portions were withdrawn and diluted 100-fold in L broth containing 250 μg of trypsin per ml to stop further colicin action. After 5 min, samples were diluted further in L broth and plated for viable counts. Colicin multiplicity (molecules of colicin per cell) was calculated from the relationship $S/S_0 = e^{-m}$, where m is the multiplicity, S_0 is the number of viable cells per milliliter before addition of colicin, and S is the number of cells per

milliliter surviving after incubation with colicin, usually for 20 min, a time after which little further killing by colicins occurred.

Measurement of the amount of cell-killing activity remaining in a cell-colicin mixture at various times after the addition of colicins was accomplished by adding suitable amounts of the mixture to 1-ml amounts of L broth containing 1.5×10^8 cells of a tetracycline-resistant indicator strain (JSM20). After exactly 20 min of incubation at 37°C, the mixtures were diluted 100-fold in L broth containing trypsin. The amount of colicin was calculated from the number of tetracycline-resistant survivors exactly as described above.

Colicin titer was sometimes determined by placing 5 μl of serial twofold dilutions of colicin or colicin-cell mix in 0.01 M sodium phosphate, pH 7.1, on lawns of indicator cells in L soft agar. The colicin titer was taken as the reciprocal of the highest dilution showing complete clearing of the lawn after incubation at 37°C.

Colicin sensitivity of various strains was determined by the halo around stab colonies. Colicinogenic strains were stabbed into LB agar plates. After overnight incubation, colicinogenic stabs were subjected to chloroform vapors and overlaid with 3 ml of LB soft agar containing 2×10^7 cells of indicator bacteria. Colicin-resistant or colicin-tolerant cells showed little or no clearing of the zone surrounding the colicinogenic stab.

Digestion of colicins and generation of fragments. Colicin products from proteolytic digestion by intact cells or outer membranes were obtained as follows. For experiments with whole cells 10^8 to 3×10^8 cells were mixed with 50 μg of purified colicin in 0.5 ml of 0.01 M sodium phosphate, pH 7.1, and the cell-colicin mix was incubated with occasional shaking at 37°C for 1 h. Cells were separated from the buffer by centrifugation in an Eppendorf centrifuge for 5 min. The supernatant was carefully decanted and precipitated with 3 volumes of ice-cold ethanol. Under these conditions, 50 to 60% of the colicin protein was recovered in the ethanol precipitate. No colicin or fragment protein could be detected in cell pellets after centrifugation. The ethanol precipitate was dissolved in a minimum amount of sample buffer (13) prior to polyacrylamide gel electrophoresis. For experiments with outer membranes, 50 μg of colicin was mixed with 100 μg of outer membrane protein in 0.5 ml of 0.01 M sodium phosphate, pH 7.1. After incubation for 1 h at 37°C, membranes were separated from the buffer by centrifugation in an Eppendorf centrifuge for 15 min. Triton-insoluble membranes were pelleted under these conditions. The supernatants from this step were treated exactly as above.

SDS-gel electrophoresis. Colicins and digestion products were analyzed by electrophoresis in the sodium dodecyl sulfate (SDS)-polyacrylamide gel system described by Laemmli (13). For the experiments reported here, 12.5% gels were prepared. Gels were stained for 2 h at 70°C by immersion in a solution containing 0.25% (wt/vol) Coomassie blue, 25% (vol/vol) isopropanol, and 10% acetic acid. Destaining was accomplished by immersing gels in 10% acetic acid–25% isopropanol at 70°C for 1 h, followed by successive washes with 10% acetic acid. In some cases gels were dried with a Bio-Rad model 224 slab gel dryer.

Transduction techniques. For mapping of *ompF*, P1 *vir* was propagated on A597, and 10^9 phage particles were mixed with 3×10^8 cells of strain KL188 (*pyrD his trp thyA rpsL*) in 1 ml of L broth containing 5 mM $CaCl_2$. *pyrD*$^+$ transductants were selected and scored for colicin A resistance by replicating to plates containing colicin A.

Protein assays. Protein was determined by the method of Lowry et al. (16), with bovine serum albumin as the standard. For membrane preparations containing Triton X-100, protein was determined in the presence of 1% SDS by the Lowry technique.

Chemicals. Triton X-100 was from Rohm and Haas Co.; *N*α-*p*-tosyl-lysylchloromethyl ketone, phenylmethylsulfonyl fluoride, and *p*-aminobenzamidine were purchased from Sigma Chemical Co.

RESULTS

Identity of the colicin produced by LK235. Comparison of the colicin produced by our culture of K235 (designated LK235) with that from a K235 culture kindly supplied by M. Nomura showed that the latter colicin conformed to the properties expected for colicin K. A mutant (A597) isolated as resistant to authentic colicin A from strain O6:H16, 23 was specifically resistant to the colicin from our LK235 culture. The mutation in A597 cotransduced with *pyrD* at a frequency of 55%, suggesting that the defect is in the *ompF* locus, a gene whose mutations produce resistance to colicin A and alter the expression of outer membrane protein 1a, which is supposedly involved in the receptor function (8; see below). As shown in Table 2, the spectrum of killing activity of the colicin from LK235 against a series of tolerant and resistant mutants of C600 further indicated that this colicin is similar, if not identical, to colicin A.

Proteolytic cleavage of colicins A and E1. Interaction of colicins A and E1 with cells results in extensive proteolysis of the colicins. Incubation of intact colicin A or E1 with sensitive cells led to cleavage of these colicins and the appearance of many fragments detectable on SDS-polyacrylamide gels. The fragments could be recovered from supernatants of cells treated with microgram amounts of colicin. In the experiment shown in Fig. 1 and in most cleavage experiments reported here, the amount of colicin added represented multiplicities of 10^5 to 10^6 colicin molecules per cell. As can be seen in Fig. 1, within 60 min of incubation at 37°C, colicin A was degraded into at least seven peptide fragments of molecular weights ranging from 20,000 to 30,000. Colicin E1 breakdown products were of smaller molecular weight, some of them electrophoresing with the solvent front. None of the observed peptides was cell derived since electrophoresis of total protein from untreated cells yielded no protein band which stained as intensely as any of the colicin fragments. Neither colicin was degraded in the absence of cells.

TABLE 2. Colicin sensitivity of indicator strains

Strains (colicin produced)	Indicator strains (genotype)[a]						
	C600	RB314 (*btuB*)	A598 (*tsx*)	A592 (*tolA*)	A593 (*tolB*)	A586 (*tolC*)	A597 (*ompF*)
JSA1(E1)	S	R	S	R	S	R	S
A737(E2)	S	R	S	R	R	S	S
A881(E3)	S	R	S	R	R	S	S
610(A)	S	pR	S	R	R	S	R
LK235(A)	S	pR	S	R	R	S	R
NO33(K)	S	S	R	R	R	S	S

[a] Colicin sensitivity was determined by the halo test described in Materials and Methods. R denotes complete resistance to the indicated colicin, pR denotes partial resistance, and S denotes sensitivity.

Several protease inhibitors were examined for their effects on the cleavage reaction. As shown in Fig. 1, *p*-aminobenzamidine, a competitive, reversible inhibitor of serine proteases (37), completely inhibited the fragmentation of colicin A, while permitting from colicin E1 the generation of some fragments larger than seen in the absence of the inhibitor. In some experiments complete conversion of intact colicin E1 to the large fragments was observed (data not shown). Arginine also inhibited the cleavage of colicin A, suggesting that protease(s) cleaves adjacent to arginine residues and has trypsin-like specificity (37). Neither tosyl-lysylchloromethyl ketone (12 mM), phenylmethylsulfonyl fluoride (6 mM), nor the chelator EDTA (10 mM) was an effective inhibitor of the cleavage of either colicin. Boiled cells did not mediate cleavage, nor was cleavage observed if unboiled cells were incubated with colicins at 0°C (data not shown). The inability of phenylmethylsulfonyl fluoride to cause complete inhibition of cleavage of colicin A is unexpected if the protease activity is similar in spectrum of action to other serine proteases (7). For the purposes of this study, it was decided to concentrate on the effect of an inhibitor, *p*-aminobenzamidine, which blocked the cleavage of either colicin at some step during proteolysis.

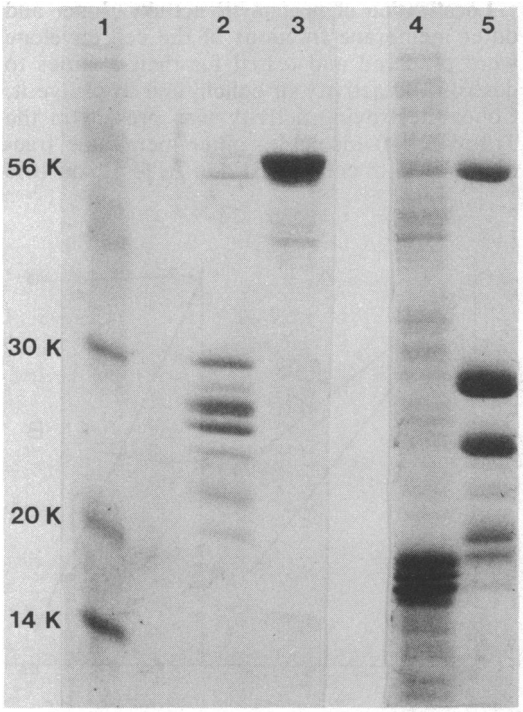

FIG. 1. Cleavage of colicins A and E1 by whole cells of C600. Fragments of colicin A or E1 were obtained as described in Materials and Methods by mixing 2×10^8 C600 cells with 50 μg of colicin protein. Lane 1, molecular weight markers: lysozyme (14,000), soybean trypsin inhibitor (20,000), carbonic anhydrase (30,000). Lane 2, supernatant of cells treated with colicin A; lane 3, supernatant of cells treated with colicin A plus 5 mM *p*-aminobenzamidine; lane 4, supernatant of cells treated with colicin E1; lane 5, supernatant of cells treated with E1 plus 5 mM *p*-aminobenzamidine. Fewer than 1% of C600 cells survived after 20 min of treatment of colicins.

The appearance of fragments in the supernatants of colicin-cell mixtures at various times after the addition of colicin was examined to detect the presence of possible intermediates. As shown in Fig. 2A, with colicin A there was a gradual decrease in the amount of intact colicin with a corresponding increase in fragments. The same series of fragments was present at each time after the addition of colicin, suggesting that no large intermediate precursor fragments were being produced. With colicin E1 the analogous experiment revealed a two-step process: two intermediate fragments of molecular weights 26,000 and 30,000 appeared first and then gave rise to the smaller fragments (Fig. 2B). The intermediate fragments of colicin E1 were identical in molecular weight to the fragments generated from colicin E1 in the presence of *p*-aminobenzamidine.

The fact that *p*-aminobenzamidine allowed fragmentation of colicin E1 to large peptides but inhibited further degradation suggests that the protease which degrades the intermediate fragments may be the same one which cleaves colicin A and that a second protease is responsible for the first fragmentation of colicin E1. The

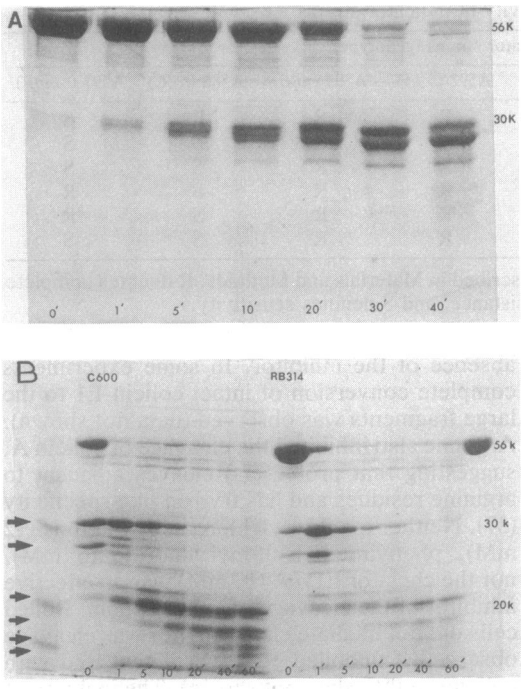

FIG. 2. Time course of appearance of fragments of colicins A and E1. (A) Colicin A (50 μg) was added to each of eight tubes containing 1.5×10^8 C600 cells in 0.5 ml of 10 mM $NaPO_4$, pH 7.1. At indicated times after colicin addition, 25 mM *p*-aminobenzamidine was added to terminate cleavage and cells were centrifuged. Supernatants containing colicin fragments were analyzed on 12.5% SDS-polyacrylamide gels. (B) Colicin E1 (50 μg) was mixed with 2×10^8 C600 or RB314 cells. At indicated times, tubes were centrifuged and supernatants were treated as in A. Left lane, molecular weight markers; right lane, untreated colicin E1. Less than 1% of input C600 cells survived after 20 min of treatment with colicins. Arrows denote the position of migration of the principal fragments of either colicin.

results also suggest that cleavage of colicin E1 into intermediate fragments exposes residues which are not accessible to protease action in the intact molecule. *p*-Aminobenzamidine retarded, but did not completely prevent, the formation of colicin E1 intermediates.

Fragments do not have cell-killing activity. The appearance of fragments in the supernatants of colicin-treated cells correlated with a loss of total cell-killing activity in the mixtures (Fig. 3). Incubation of either colicin with sensitive cells led to a time-dependent disappearance of killing activity. *p*-Aminobenzamidine prevented the disappearance of colicin A activity completely and that of colicin E1 activity only partially. The protection of colicin A activity by *p*-aminobenzamidine points to proteolysis as the cause of loss of activity and suggests that fragments are inactive against cells. That *p*-aminobenzamidine only retarded the loss of colicin E1 activity correlates with the partial inhibition of fragmentation of this colicin by *p*-aminobenzamidine (Fig. 1). Colicin E1 intermediate fragments are apparently inactive, as are the products of further steps in proteolysis.

Proteolysis does not result in colicin activation. To determine whether proteolysis of these colicins is a step in their lethal action, killing by colicins was measured in the presence of *p*-aminobenzamidine (Fig. 4). The rate and extent of killing by either colicin A or E1 were actually higher in the presence of *p*-aminobenzamidine than in its absence. This suggests that *p*-aminobenzamidine, by inhibiting cleavage, causes the preservation of a greater number of intact, active colicin molecules. This is especially clear for colicin A, whose fragmentation was completely prevented by *p*-aminobenzamidine.

Localization of proteolytic activity. Inner and outer membrane fractions of the cell envelope were prepared and tested for their abilities to destroy the activity of colicin and to cleave it. Colicin-destroying activity was present in the Triton X-100–insoluble, outer membrane fraction. For both colicins E1 and A, less than 10%

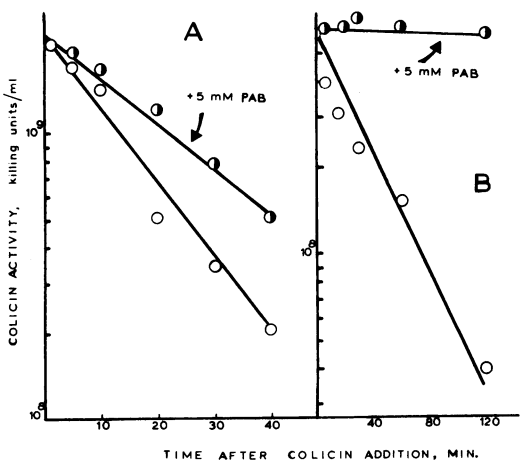

FIG. 3. Loss of colicin activity caused by exposure to cells of strain C600. (A) Colicin E1 (10 ng; 2×10^9 killing units) was mixed with 10^9 C600 cells in 1 ml of 0.01 M $NaPO_4$, pH 7.1. The amount of colicin activity remaining in the colicin-cell mixture at times after colicin addition was calculated as described in Materials and Methods by measuring viability of strain JSM20. Symbols: ○, no *p*-aminobenzamidine; ◑, 5 mM *p*-aminobenzamidine present with cells. (B) Colicin A (100 ng; 6×10^8 killing units) was mixed with 6×10^8 C600 cells in 1 ml of $NaPO_4$. Symbols as in A. Survival of C600 was less than 10% after 120 min of colicin A treatment.

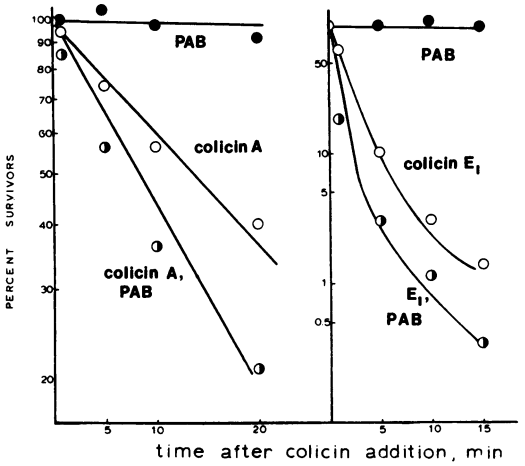

FIG. 4. Effect of *p*-aminobenzamidine on killing by colicins. (A) At 0 min, 0.1 μg of colicin A was mixed with 1.6×10^8 cells of C600 per ml in a volume of 1 ml. At the indicated times, 0.1 ml was withdrawn and diluted 100-fold into L broth containing 250 μg of trypsin per ml to stop colicin action. After 5 min of digestion of trypsin, cells were further diluted in L broth and plated for colony-forming ability. Symbols: ○, colicin A alone; ◑, colicin A plus 5 mM *p*-aminobenzamidine; ●, 5 mM *p*-aminobenzamidine, no colicin. (B) As in A except that 15 ng of colicin E1 was mixed with 1.4×10^8 cells of C600 per ml.

of original colicin activity was present after a 10-min incubation with 100 μg of outer membrane protein, whereas 60% of the colicin activity was measurable after 10 min of incubation with 100 μg of Triton-soluble membrane protein. Similar results were obtained with membrane fraction prepared by sucrose density gradient centrifugation. Outer membranes prepared by either technique mediated complete fragmentation of the colicins within 30 min of incubation at 37°C, whereas the gradient-prepared inner membrane fraction digested less than 50% of the total amount of colicin protein (data not shown). The series of fragments generated by incubation of either colicin with outer membranes was the same as that generated by incubation with whole cells (data not shown).

Interaction of colicin A with cells in the absence of proteolysis. The fact that *p*-aminobenzamidine completely inhibits breakdown of colicin A makes it possible to explore the question of why the killing titer of colicin preparations is usually 10- to 1,000-fold lower than the number of molecules (18, 23). If breakdown of most of the colicin is a cause of this discrepancy, then preventing breakdown might bring the two values together.

When mixtures of colicin A and sensitive bacteria (incubated in the presence of *p*-aminobenzamidine) were added to tetracycline-resistant indicator bacteria, the killing titer did not diminish with time (Fig. 3B). In this experiment, 6×10^8 "killing units" of colicin A (corresponding to about 6×10^{10} molecules) interacted with 6×10^8 cells, killing 80% of them after 1 h. With the assumption that one molecule of colicin is sufficient to kill one cell (23, 29), a minimum of 5×10^8 colicin molecules consumed in killing 5×10^8 cells must be a small fraction of the number of molecules present and capable of killing cells, implying that there are many more active molecules than are scored as "killing units." When the mixtures were centrifuged and supernatant and resuspended pellet were tested separately for killing, almost all colicin activity proved to be in the resuspended pellet (Table 3). Under conditions that prevent colicin proteolysis, colicin molecules were rapidly removed from the medium; a small fraction of them acted lethally, but most molecules remained attached to the bacteria in such a way that they cosedimented with cells while remaining available to desorb and kill other bacteria. The possibility of such a reversible colicin-cell interaction has already been suggested by Shannon and Hedges (36).

Involvement of specific colicin receptors in colicin cleavage. Receptor-deficient mutants were tested for their abilities to inactivate and cleave

TABLE 3. Adsorption of colicin A by C600 and A597[a]

Prepn	Relative colicin titer[b]	
	C600	A597[c]
Mixture	1,024	1,024
Supernatant	256	1,024
Resuspended pellet	512	32
Supernatant of resuspended pellet	32	32

[a] Colicin A (4 μg; approximately 2×10^{10} killing units) was mixed with 6×10^8 cells of either C600 or the *ompF* mutant (A597) in 1 ml of 10 mM sodium phosphate, pH 7.1, containing 5 mM *p*-aminobenzamidine. Cell mixtures were incubated for 2 min at 37°C, assayed for colicin activity by spot testing and centrifuged. The supernatants from each mixture were assayed, and the cell pellet was resuspended in an appropriate volume of buffer containing 5 mM *p*-aminobenzamidine. After 2 min of incubation at 37°C, colicin present in each resuspended mix was assayed, as was the colicin activity present in the supernatant of the pellet after centrifugation. The indicator strain was JSM20.

[b] Reciprocal of dilution of colicin showing complete clearing of the lawn.

[c] Cells of C600 and A597 were grown in L broth medium lacking added NaCl, conditions under which a maximum amount of protein 1a is present in the outer membrane (31).

colicins. It is well established (6, 43) that the outer membrane receptor for E-type colicins requires a functional *btuB* gene product, an outer membrane protein also involved in the binding and transport of vitamin B_{12}. As shown in Fig. 5, cells of the wild type (C600) and the receptor-deficient *btuB* mutant (RB314) inactivated colicin E1 at similar rates. Likewise, the rate of appearance of colicin E1 fragments was largely unaffected by the *btuB* mutation (Fig. 2B). When cell-colicin mixtures were centrifuged and the supernatants were assayed for residual colicin activity, between 66 and 90% of colicin E1 activity disappeared from the supernatants of wild-type cells, whereas little or no activity was removed by the *btuB* cells (Fig. 5B). Thus, it appears that proteolysis is not greatly affected by mutational loss of colicin E1 receptors, an indication that receptors have a nonessential role in cleavage. Colicin E1 can be bound by wild-type cells but not by receptor-deficient cells.

Analogous conclusions could be drawn for colicin A (Table 3). When colicin A was incubated with *ompF* cells for several minutes in the presence of *p*-aminobenzamidine (which prevents fragmentation), followed by centrifugation, most colicin activity was present in the supernatant, and little was recoverable from the resuspended pellet.

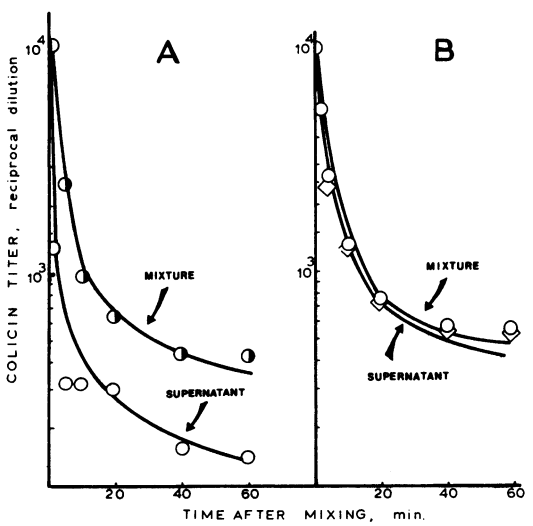

FIG. 5. Destruction of colicin E1 by cells of C600 or RB314. Colicin E1 (25 μg) was mixed with 2×10^8 C600 or RB314 cells in a series of centrifuge tubes containing 0.5 ml of 0.01 M $NaPO_4$, pH 7.1. At the indicated times the cell-colicin mixture was titrated by spot testing on JSM20 and centrifuged. The supernatant fluids were also titrated. (A) C600 cells: ◑, titer before centrifugation; ○, titer after centrifugation. (B) RB314 cells; ○, titer before centrifugation. ◇, titer after centrifugation.

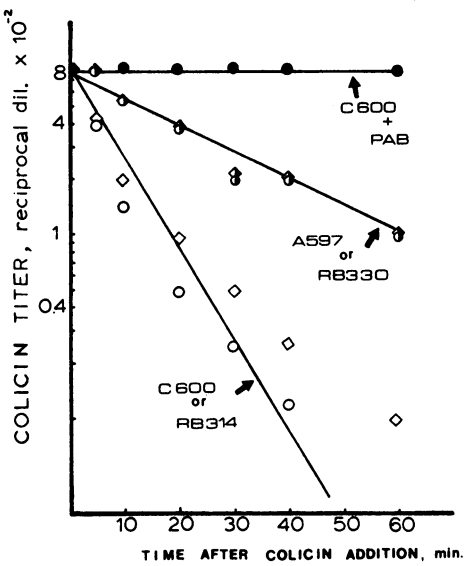

FIG. 6. Destruction of colicin A activity by outer membranes. Colicin A (0.5 μg) was mixed with outer membranes (100 μg protein) from C600 (○), RB314 (◇), A597 (◑), or RB330 (◈) in 1 ml of 0.01 M $NaPO_4$, pH 7.1. At the indicated times, the colicin-membrane mix was diluted and assayed for activity by spot testing on strain JSM20. As a control, colicin was mixed with outer membranes of C600 in the presence of 5 mM *p*-aminobenzamidine (●). More than 95% of the total succinate dehydrogenase activity was present in the Triton-soluble fraction and more than 95% of total cell wall 2-keto-3-deoxyheptonic acid was associated with the Triton-insoluble outer membranes, implying very little cross-contamination with inner membrane.

Although adsorption of colicin A is defective in *ompF* mutants, proteolysis still occurs. Whole cells of *ompF* mutants cleaved and inactivated colicin A more slowly than did wild-type cells. There was no difference in the rates of cleavage and inactivation of colicin E1 (data not shown). Consistent with the observation with whole cells, outer membranes from *ompF* mutants cleaved colicin A more slowly than did membranes from the wild type. Colicin A was detectable in supernatants of colicin-treated outer membranes from *ompF* cells, whereas during the same incubation period it disappeared from supernatants of membranes from the wild type or *btuB* mutants (data not shown). The rate of inactivation of the cell-killing activity of colicin A by outer membrane paralleled the kinetics of fragment production, indicating that lower rates of inactivation and cleavage of colicin A are due to the *ompF* mutation (Fig. 6). Taken together, the findings with resistant mutants indicate that mutational loss of receptors does not cause a loss of colicin-cleaving activity, although at least

with respect to colicin A the mutation may alter the rate of colicin cleavage and inactivation.

DISCUSSION

It is apparent that proteolysis of colicins can be one of the outcomes of their interaction with components of the *E. coli* cell surface. Proteolysis of various colicins has been demonstrated by several over groups. To account for the failure of resistant mutants to cleave colicin E4, Cavard and Lazdunski (2) proposed a model in which binding of colicin to specific receptors triggered a protease activity which could cleave the colicin. These authors suggested the possibility that cleavage of the colicin is necessary as a prelude to the lethal event. (An "activated fragment" model for colicin action was put forward by Watson and Sherratt [40]. The data supporting this model have been retracted [J. Sherratt, personal communication].) A mechanism in which cleavage is essential for the lethal action of colicin would imply that an "activated fragment" would be the result of proteolysis, as has been demonstrated for the action of diphtheria toxin on mammalian cells (9). Bowles and Konisky (1) have shown that membranes from either the wild type or resistant mutants mediated cleavage of colicin Ia, suggesting that specific receptors may not be involved in the proteolytic reaction.

Experiments reported here included a detailed analysis of the proteolysis of colicins A and E1. For both colicins there is evidence that proteolysis is unrelated to any mechanism of colicin killing since an inhibitor of proteolysis increased colicin activity. Experiments with the protease inhibitor *p*-aminobenzamidine suggest that although some molecules participate in cell killing most of the colicin molecules adsorbed to sensitive cells do not act as killers. That only some colicin receptors are able to mediate cell killing has been suggested repeatedly (10, 12, 36).

Although it is probable that extensive proteolysis of colicins A and E1 does not produce fragments involved with cell killing, it cannot be ruled out that a separate proteolytic event might be part of the killing mechanism. For colicin A one would have to assume that such a proteolytic event is insensitive to inhibition by *p*-aminobenzamidine. Even if one or more of the fragments derived from the interaction of colicin A with cells turns out to have some biochemical activity, it would not necessarily implicate such fragments in killing by colicins. Fragmentation of some colicins and bacteriocins into moieties with biochemical action has been found to result from digestion by certain proteases (4, 25).

Bacterial mutants that fail to adsorb colicin E1 or A can still give rise to proteolytic fragments, suggesting that receptor function is not required for colicin cleavage. Thus, proteolysis of either colicin still occurs in the absence of receptor activity, supporting the notion that events leading to killing are separate from interactions leading to colicin breakdown. Receptors, when present, may make colicin more accessible to protease enzymes.

A traditional view would hold that receptors behave purely as colicin binders (3, 18, 20, 23, 29, 30). Hence, decrease of free colicin activity in the presence of sensitive cells or isolated membrane components has been taken as a measure of binding to specific receptors. Clearly, proteolysis of colicins can mask receptor activity. The fact that specific adsorption of colicin A by sensitive cells occurs in the absence of proteolysis provides evidence that receptor activity is distinct from proteolytic activity.

The relationship between the activities directed against colicins A and E1 and other protease activities associated with the outer membrane is not clear. The preponderance of anti-colicin activity in the outer membrane may distinguish it from leader peptidase activity which processes M13 procoat protein (19). Whether this activity is related to proteolytic activity which degrades abnormal proteins (21) or to the one which attacks inner membrane nitrate reductase (17) is also unknown. Outer membrane proteases may have broad specificity. In a recent report, Leytus et al. (15) showed that outer membranes contain a protease activity which activates plasminogen to plasmin, presumably an activity also responsible for cleaving colicin Ia.

If considerable inactivation of colicins occurs even with receptor-deficient mutants, how can such strains in the past have been classified as resistant by a variety of tests of colicin adsorption (3, 18, 20, 23, 29, 30)? The question may have several answers. Buffer conditions probably affect the amount of colicin inactivated: the small difference between the wild type and the *btuB* mutant in colicin E1 inactivation assayed in sodium phosphate was amplified when assayed in L broth, suggesting that ingredients such as small peptides, amino acids, or ions affect the degree of proteolysis or colicin binding (unpublished data). In addition, not all colicins may be substrates for bacterial protease(s): preparations of colicin E2 are not cleaved by cells or outer membranes (unpublished data), and colicin Ia is not cleaved by whole cells (1). Any experimental protocol designed to purify receptors from membranes should take into account possible colicin proteolysis.

ACKNOWLEDGMENTS

Research reported in this paper was supported by grants to S. E. Luria from the National Science Foundation (PCM-7614956) and the National Institute of Allergy and Infectious Diseases (Public Health Service grant 5-R01-AI03038).

I thank S. E. Luria, J. Suit, V. Clark, and C. Kayalar for helpful discussion. Thanks are due to M. Colten for typing the manuscript.

LITERATURE CITED

1. **Bowles, L. K., and J. Konisky.** 1981. Cleavage of colicin Ia by the *Escherichia coli* K-12 outer membrane is not mediated by the colicin Ia receptor. J. Bacteriol. **145**:668–671.
2. **Cavard, D., and C. Lazdunski.** 1979. Interaction of colicin E4 with specific receptor sites mediates its cleavage into two fragments inactive towards whole cells. Eur. J. Biochem. **96**:525–533.
3. **Davies, J. K., and P. Reeves.** 1975. Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group A. J. Bacteriol. **123**:102–117.
4. **DeGraaf, F. K., M. J. Stukart, F. C. Boogerd, and K. Metselaar.** 1978. Limited proteolysis of cloacin DF 13 and characterization of the cleavage products. Biochemistry **17**:1137–1142.
5. **Diedrich, D. L., A. O. Summers, and C. A. Schnaitman.** 1977. Outer membrane proteins of *Escherichia coli*. V. Evidence that protein 1 and bacteriophage-directed protein 2 are different polypeptides. J. Bacteriol. **131**:598–607.
6. **Dimasi, D. R., J. C. White, C. A. Schnaitman, and C. Bradbeer.** 1973. Transport of vitamin B_{12} in *Escherichia coli*: common receptor sites for vitamin B_{12} and the E colicins on the outer membrane of the cell envelope. J. Bacteriol. **115**:501–513.
7. **Fahrney, J. E., and A. M. Gold.** 1963. Sulfonyl fluorides as inhibitors of esterases. J. Am. Chem. Soc. **85**:997–1000.
8. **Foulds, J., and T-j. Chai.** 1979. Isolation and characterization of isogenic *E. coli* strains with alterations in the level of one or more major outer membrane protein. Can. J. Microbiol. **25**:423–427.
9. **Gill, D. M.** 1978. Seven toxic peptides that cross cell membranes, p. 291–332. *In* J. Jeljaszewicz and T. Wadstrom (ed.), Bacterial toxins and cell membranes. Academic Press, Inc., New York.
10. **Kadner, R. J., and P. J. Bassford.** 1978. The role of the outer membrane in active transport, p. 413–462. *In* B. P. Rosen (ed.), Bacterial transport. Marcel Dekker Inc., New York.
11. **Kayalar, C., and S. E. Luria.** 1979. Channel formation by colicin K on liposomes, p. 297–306. *In* C. P. Lee, G. Schatz, and L. Ernster (ed.), Membrane bioenergetics. Addison-Wesley Publishing Co., Reading, Mass.
12. **Konisky, J.** 1979. Specific transport systems and receptors for colicins and phages, p. 319–359. *In* M. Inouye (ed.), Bacterial outer membranes: biogenesis and functions. John Wiley & Sons, Inc., New York.
13. **Laemmli, U.** 1970. Cleavage of structural proteins during the assembly the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lau, C., and F. M. Richards.** 1976. Behavior of colicins E1, E2, and E3 attached to sephadex beads. Biochemistry **15**:666–671.
15. **Leytus, S. P., L. K. Bowles, J. Konisky, and W. F. Mangel.** 1981. Activation of plasminogen to plasmin by a protease associated with the outer membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **78**:1485–1489.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. **MacGregor, C. H., C. W. Bishop, and J. E. Blech.** 1979. Localization of proteolytic activity in the outer membrane of *Escherichia coli*. J. Bacteriol. **137**:574–583.
18. **Maeda, A., and M. Nomura.** 1966. Interaction of colicins with bacterial cells. I. Studies with radioactive colicins. J. Bacteriol. **91**:85–694.
19. **Mandel, G., and W. Wickner.** 1979. Translational and post-translational cleavages of M13 procoat protein: extracts of both cytoplasmic and outer membranes of *Escherichia coli* contain leader peptidase activity. Proc. Natl. Acad. Sci. U.S.A. **76**:236–240.
20. **Manning, P. A., and P. Reeves.** 1978. Outer membrane proteins of *Escherichia coli* K-12: isolation of a common receptor protein for bacteriophage T6 and colicin K. Mol. Gen. Genet. **158**:279–286.
21. **Murakami, K., R. Voellmy, and A. L. Goldberg.** 1979. Protein degradation is stimulated by ATP in extracts of *Escherichia coli*. J. Biol. Chem. **254**:8194–8200.
22. **Nagel de Zwaig, R., and S. E. Luria.** 1967. Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. J. Bacteriol. **94**:1112–1123.
23. **Nomura, M.** 1967. Colicins and related bacteriocins. Annu. Rev. Microbiol. **21**:257–284.
24. **Nomura, M. J. Sidikaro, K. Jakes, and N. Zinder.** 1974. Effects of colicin E3 on bacterial ribosomes, p. 805–813. *In* M. Nomura, A. Tissieres, and P. Lengyel (ed.), Ribosomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Ohno, S., Y. Ohno-Iwashita, K. Suzuki, and K. Imahori.** 1977. Purification and characterization of active component and active fragment of colicin E3. J. Biochem. (Tokyo) **83**:1045–1053.
26. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. **247**:3962–3972.
27. **Plate, C. A.** 1979. Requirement for membrane potential in active transport of glutamine by *Escherichia coli*. J. Bacteriol. **137**:221–226.
28. **Pugsley, A. P., and C. A. Schnaitman.** 1978. Outer membranes of *Escherichia coli*. VII. Evidence that bacteriophage-directed protein 2 functions as a pore. J. Bacteriol. **133**:1181–1189.
29. **Reeves, P.** 1972. The bacteriocins. Springer-Verlag, New York.
30. **Sabet, S., and C. A. Schnaitman.** 1973. Purification and properties of the colicin E3 receptor of *Escherichia coli*. J. Biol. Chem. **248**:1797–1806.
31. **Sato, T., and T. Yura.** 1981. Regulatory mutations conferring constitutive synthesis of major outer membrane proteins (OmpC and OmpF) in *Escherichia coli*. J. Bacteriol. **145**:88–96.
32. **Schaller, K., and M. Nomura.** 1976. Colicin E2 is a DNA endonuclease. Proc. Natl. Acad. Sci. U.S.A. **73**:3989–3995.
33. **Schein, S. J., B. L. Kagan, and A. Finkelstein.** 1978. Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membranes. Nature (London) **276**:159–162.
34. **Schnaitman, C. A.** 1974. Outer membrane proteins of *Escherichia coli*. IV. Differences in outer membrane proteins due to strain and cultural differences. J. Bacteriol. **118**:454–464.
35. **Schwartz, S. A., and D. R. Helinski.** 1971. Purification and characterization of colicin E1. J. Biol. Chem. **246**:6318–6327.
36. **Shannon, R., and A. J. Hedges.** 1973. Reversibility of the specific adsorption of colicin E2-P9 to cells of colicin-sensitive strains of *Escherichia coli*. J. Bacteriol. **116**:1136–1144.
37. **Stroud, R. M., M. Krieger, R. E. Koeppe, A. A. Kossiakoff, and J. L. Chambers.** 1975. Structure-function relationships in the serine proteases, p. 13–32. *In* E. Reich, D. B. Rifkin, and E. Shaw (ed.), Proteases and biological control. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Tokuda, H., and J. Konisky.** 1978. *In vitro* depolarization of *Escherichia coli* membrane vesicles by colicin Ia. J. Biol. Chem. **253**:7731–7737.
39. **Toduka, H., and J. Konisky.** 1979. Effect of colicins Ia and Ib on ion permeability of liposomes. Proc. Natl. Acad. Sci. U.S.A. **76**:6167–6171.
40. **Watson, D. H., and D. J. Sherratt.** 1979. *In vivo* proteolytic cleavage of colicins requires specific receptor binding. Nature (London) **278**:362–364.

41. **Weiss, M. J., and S. E. Luria.** 1978. Reduction of membrane potential, an immediate effect of colicin K. Proc. Natl. Acad. Sci. U.S.A. **75**:2483–2487.
42. **Weissbach, A., and J. Hurwitz.** 1958. The formation of 2-keto-3-deoxyheptonic acid in extracts of *Escherichia coli* B. J. Biol. Chem. **234**:705–709.
43. **White, J. C., P. M. DiGirolamo, M. L. Fu, Y. A. Preston, and C. Bradbeer.** 1973. Transport of vitamin B_{12} in *Escherichia coli*: location and properties of the initial B_{12}-binding site. J. Biol. Chem. **248**:3978–3986.