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Structural and Functional Properties of Colicin M

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Colicin M of *Escherichia coli* C1139 was isolated in pure form. It consisted of a single polypeptide with a molecular weight of 27,000 ± 2,000. Colicin M lysed sensitive cells of *E. coli* but had to act continuously up to the point when lysis commenced (after 20 min). Colicin M was largely resistant to hydrolysis by trypsin except when adsorbed to cells. Within 4 to 5 min after addition of colicin M, cells could be rescued by trypsin or sodium dodecyl sulfate. Later, colicin M was apparently inaccessible to these inactivating agents. Killing of cells by colicin M required Ca^{2+} ions. Cells could be rescued with ethylene glycol-bis(β-amino-ethyl ether)-*N,N'*-tetraacetate (EGTA) immediately before the onset of lysis. Under these conditions, colicin M remained bound to the cells, and it became again sensitive to trypsin. We conclude that under the influence of EGTA colicin M is removed from its site of action and becomes again accessible to trypsin at the cell surface.

Colicin M was originally defined on the basis that resistant cells were cross-resistant to phages T1 and T5 (6). Later it was shown that colicin M causes lysis of sensitive cells (2). Both observations were of interest since the uptake of the toxic protein required the function coded by the *tonA* (now *fhua* [5, 11]) and *tonB* genes, and lysis of cells as a primary event was unique among the actions of the known colicins. Cells treated with colicin M formed spheroplasts under osmotic protection similar to those observed with penicillin. Electron microscope examinations revealed that at first bulges appeared in the cell envelope, most frequently equatorially but also at sites distributed all over the cell (2). When sucrose was added to a rapidly growing culture of *Escherichia coli*, lysis was delayed for about 40 min but not prevented. Earlier attempts to demonstrate hydrolysis of the isolated murein remained ambiguous since the isolation of pure colicin M was hampered by the low synthesis of the producing strain, which could not be induced with mitomycin C (2). In addition, colicin M remained firmly bound to the cells, so it was necessary to purify it from a total cell homogenate. It was therefore possible that murein hydrolases present in the colicin M preparations as contaminants were responsible for the frequently observed murein hydrolysis.

One of us (K.S.) observed during his stay in the laboratory of M. J. Osborn that *E. coli* C1139 produced much larger amounts of colicin M than did *E. coli* 32T19F, the strain used previously. However, this strain also synthesized colicin B (8), which had to be separated from colicin M. In this paper, we report the isolation of pure colicin M and describe some of its properties.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. Table 1 lists the *E. coli* strains used. Colicin-sensitive cells were grown at 37°C in a medium containing 1% tryptone, 0.5 yeast extract (both from Difco Laboratories, Detroit, Mich.), and 1% NaCl. For agar plates, 1.5% agar (Difco) was added. The absorbance of cell cultures was measured at 578 nm with an Eppendorf photometer (0.1 corresponded to 10^8 cells/ml).

Induction of colicin M. A 500-ml sample of an overnight culture of *E. coli* C1139 was used to inoculate 200 liters of sterile M9 minimal medium (1) supplemented with 0.6% Casamino Acids (Difco) and 1% glucose, prewarmed to 37°C. The culture was aerated with 2,000 liters of air per h. At a cell density of 3×10^8 per ml, mitomycin C (Sigma Chemical Co.; St. Louis, Mo.; 0.2 mg/liter) and antifoam (Serva, Heidelberg, West Germany; 0.1 ml/liter) were added. Aeration was continued for another 3 h. Then the cells were chilled to 5°C with ice and harvested in a Sharples centrifuge. Harvesting of the 200-liter culture took approximately 150 min. The yield of wet cells was 1,500 g. The cells were frozen in liquid nitrogen.

Colicin assay and protein determination. A spot assay similar to that described by Goebel and Barry (7) was used. Tryptone-yeast extract agar plates were overlaid with 3 ml of soft tryptone-yeast extract agar (0.7% agar) containing approximately 10^8 indicator bacteria. Samples of 10 μl of different colicin dilutions in tryptone-yeast extract medium were spotted on the overlay. The plates were incubated overnight

TABLE 1. *Bacterial strains*

E. coli strain	Genotype and comments	Col factor	Source or reference
K-12			
32T19F	*met rpsL tonA*, produces colicin M	ColM-K260	P. Fredericq
Cl139	*leu lac thi*, produces colicins B and M	ColB, ColM	S. Guterman
AB2847	*aroB thi tsx malT*, sensitive to the colicins B and M		B. Bachmann
IR20	Derivative of AB2847 *fepA*, resistant to colicin B		1
P8	Derivative of AB2847 *fhuA*, resistant to colicin M		1

at 37°C. The colicin titer was expressed as the reciprocal of the final dilution which gave a clear spot. A more quantitative assay related the lethal units of colicin M to the mass of protein (see Results). Protein was assayed with Fluram as described by the supplier (Hoffmann-La Roche, Inc.), using bovine serum albumin as the standard.

Isolation of colicin M. All operations were performed at 4°C. Frozen wet cells of *E. coli* Cl139 (200 g) were suspended in 400 ml of distilled water containing 1 μg of crude DNase per ml (DN-25; Sigma) and disrupted by sonication. Cell debris was centrifuged for 4 h at 27,000 × *g*. From the turbid supernatant, a sample was dialyzed against water and assayed for colicin activity and protein concentration. The pH of the supernatant was slowly adjusted to pH 5.2 with 2 M sodium acetate, pH 5.1. The supernatant and precipitate were separately collected after centrifugation for 30 min at 27,000 × *g*. The precipitate contained 20 to 50% of the colicin activity. It was suspended in 100 ml of cold 10 mM sodium phosphate buffer, pH 6, once more titrated to pH 5.2, and centrifuged. The pooled turbid supernatant contained the colicin B and M activity.

Column chromatography on carboxymethyl cellulose (Whatman CM 52). The pooled supernatants were dialyzed four times against 10 liters of the column equilibration buffer (10 mM sodium phosphate, pH 6.0, containing 1 mM dithiothreitol and 0.02% sodium azide). Parallel chromatographies were performed with and without 0.1% Triton X-100 in the elution buffers. The colicin solution was clarified by centrifugation (1 h at 27,000 × *g*) and applied to the column (3.5 by 75 cm). Fractions (9 ml/h) were collected in polypropylene tubes and monitored for absorbance, salt concentration, and colicin activity. After extensive washing with 3 liters of buffer, colicin M had remained on the column, whereas colicin B had been eluted. Then 10 liters of a linear gradient consisting of 0.0 to 0.3 M NaCl in the equilibration buffer was applied. Colicin M was eluted after approximately 4.5 liters. Fractions containing high colicin activity were stored in the tubes at 4°C.

Amino acid analyses. Samples of colicin M were extensively dialyzed against double-distilled water and then freeze-dried. They were hydrolyzed with distilled 5.7 N HCl under nitrogen for 18 h at 105°C. For the determination of cysteine as cysteic acid and of methionine as methionine sulfone, freeze-dried samples were oxidized with performic acid (10). The analyses were performed on a Biotronic amino acid analyzer LC 6000 E according to the supplier's program.

Gel electrophoresis. The purity of the colicin M preparations was tested by electrophoresis on polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate with and without 6 M urea, with urea alone, or without any denaturing agent. The procedure followed essentially the method described by Lugtenberg et al. (14). Furthermore, electrofocusing experiments were performed on polyacrylamide gels according to the technique prescribed by the supplier of the apparatus and the ampholines used (LKB Instruments, Gräfelfing, West Germany) or according to Vesterberg (20). The molecular weight of colicin M was estimated by comparing its electrophoretic mobility with those of the standards ovalbumin, chymotrypsinogen, and cytochrome *c*.

Enzyme assays. Protease activity was determined with azocasein (Serva) as described previously (4). The lipase activity of pure colicin M was tested with [^{14}C]acetate-labeled sensitive cells or with labeled phospholipids. Release of fatty acids was tested by thin-layer chromatography on silica gel plates with the solvent chloroform-methanol-acetic acid (65:25:8, vol/vol/vol) or by gas chromatography of fatty acid methyl esters. Glycosidase and amidase activity was tested with isolated [^{3}H]diaminopimelate-labeled murein and with [^{14}C]*N*-acetylglucosamine-labeled murein and lipopolysaccharide by measuring the release of material soluble in 5% trichloroacetic acid.

RESULTS

Mitomycin induction of colicin M. Colicin synthesis can usually be induced with mitomycin C in Col factor-carrying strains of *E. coli*. However, we did not find significant induction of colicin M in *E. coli* 32T19F (2), but in a culture of *E. coli* Cl139, production of colicins M and B was increased 500- to 1,000-fold with mitomycin C. Cells treated with 1 μg of mitomycin C per ml began to lyse 150 min after the addition of the drug (microscopic observation and turbidity measurements). About half of the colicin B and M activities sedimented with the cell pellet, which had a titer of 500,000 arbitrary dilution units per g of wet cells. Both colicins were released from the cells upon sonication. In con-

trast, extraction of colicins M and B from intact cells with 1 M NaCl yielded only 5 to 10% of the sedimenting activity. Less lysis occurred with 0.2 μg of mitomycin C per ml. The amount of sedimenting activity remained the same, whereas the soluble fraction contained only 20% of the total activity. We used only 0.2 μg of mitomycin C per ml for large-scale preparations and isolated colicin M from the cells.

Purification of colicin M. The procedure for purifying colicin M was essentially the same as previously reported (2). A representative preparation with the modifications used is described in Materials and Methods and summarized in Table 2. Colicin M was purified to apparent homogeneity by a two-step procedure. After sonication of the cells and separation of the cell debris and most of the membrane fraction by centrifugation, the colicin M and B activities were found in the supernatant. Precipitation at pH 5.2 removed about half of the contaminating protein. The colicin activities remained in the turbid supernatant. The supernatant was extensively dialyzed and clarified by centrifugation. The clear solution containing all of the colicin B and M activities was applied to a carboxymethyl cellulose column. Colicin M was bound to the cation-exchange resin, whereas colicin B and most of the contaminating proteins were not retarded (Fig. 1). This property of colicin B is in agreement with that described by Pugsley and Reeves (18), who purified colicin B on a diethylaminoethyl cellulose column. Good purification of colicin M was dependent on a slow flow rate and on extensive washing with the buffer used to equilibrate the resin. The colicin was eluted by raising the ionic strength of the elution buffer with NaCl. Of importance was the use of a flat salt gradient and the inclusion of dithiothreitol in the elution buffer.

Without dithiothreitol and with a faster elution rate, the colicin M-containing fractions were contaminated with two enzymatic activities, a protease and an endopeptidase. The protease was recognized when colicin M was chromatographed on a column of Bio-Gel P-150 (Bio-Rad Laboratories, Munich) in the presence of 6 M urea. Upon standing, the second half of the colicin M peak was degraded. The endopeptidase activity was seen when colicin M was added to a mixture of murein degradation products, obtained by hydrolysis of isolated murein-lipo-

TABLE 2. *Purification of colicin M from E. coli C1139 (ColB, ColM)*

Fraction	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)	Purification factor
Cell-free extract (from 200 g)	400	18,000	4×10^7	2.2×10^3	100	
Pooled acetate supernatants	660	9,240	4×10^7	4.3×10^3	100	2
Pooled peak fraction of column chromatography	65	78	3×10^7	4×10^5	75	182

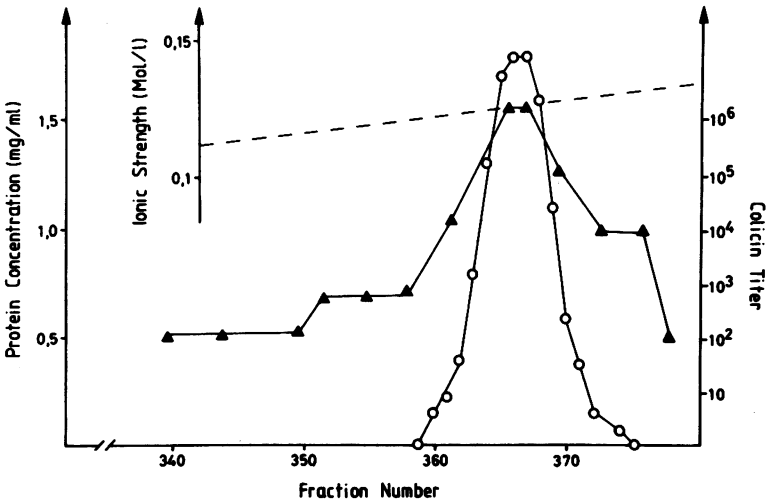

FIG. 1. *Purification of colicin M in the presence of 1% Triton X-100 on a carboxymethyl cellulose column. More than 90% of the protein, including colicin B, was eluted with the first 50 fractions (not shown). Only the section of the chromatogram where colicin M was eluted is shown. Symbols:* (○) *Protein concentration;* (▲) *colicin M activity. Inset* (——), *ionic strength of the eluent determined by measuring conductivity.*

protein complex with lysozyme (5). Upon incubation, the dimer of the murein subunits, cross-linked between D-alanine and *meso*-diaminopimelic acid, was converted to the monomeric subunit. The products were not characterized chemically, but the compound at the position of the dimer disappeared and showed up at the position of the monomer after paper chromatography with the system isobutyric acid–1 M ammonium hydroxide (5:3, vol/vol) (5). The same system revealed that the amount of murein subunits remaining bound to the lipoprotein stayed constant, which argues against an amidase activity in the colicin M preparation. We also found no phospholipase, glycosidase, or protease activity in the pure colicin M preparation used throughout these studies.

Triton X-100 was added to the chromatography buffer after it had been recognized that colicin M remained stable in a Triton-containing solution (see below). The detergent did not alter the purification of colicin M on the column.

The purified protein showed one major band after polyacrylamide gel electrophoresis (Fig. 2) with an electrophoretic mobility slightly slower than that of chymotrypsinogen, indicating an apparent molecular weight of 27,000. This figure agrees well with the value found by gel filtration: the experimental V_e/V_o value on Sephadex G-100 corresponded to an apparent molecular weight of 27,000 ± 2,000 (2). The isoelectric point was determined by isoelectric focusing on polyacrylamide gels. Colicin M showed a single peak in a position of about pH 9.5, which is in agreement with its strong binding to carboxymethyl cellulose. The colicin M preparations sometimes contained a minor protein band which moved slightly behind the dye front in gels with sodium dodecyl sulfate (Fig. 2) and which showed an isoelectric point very similar to that of colicin M. However, the same protein was obtained when a parallel preparation was run in exactly the same way through all the steps of the colicin M isolation procedure with *E. coli* IR20, which does not bear a Col plasmid. This protein is therefore not an immunity protein (12) for colicin B or M. It also does not participate in the action of colicin M, since we obtained colicin M preparations which were free of this protein.

Colicin M isolated with the modified procedure was free of phosphatidylethanolamine since after acid hydrolysis no ethanolamine was ob-

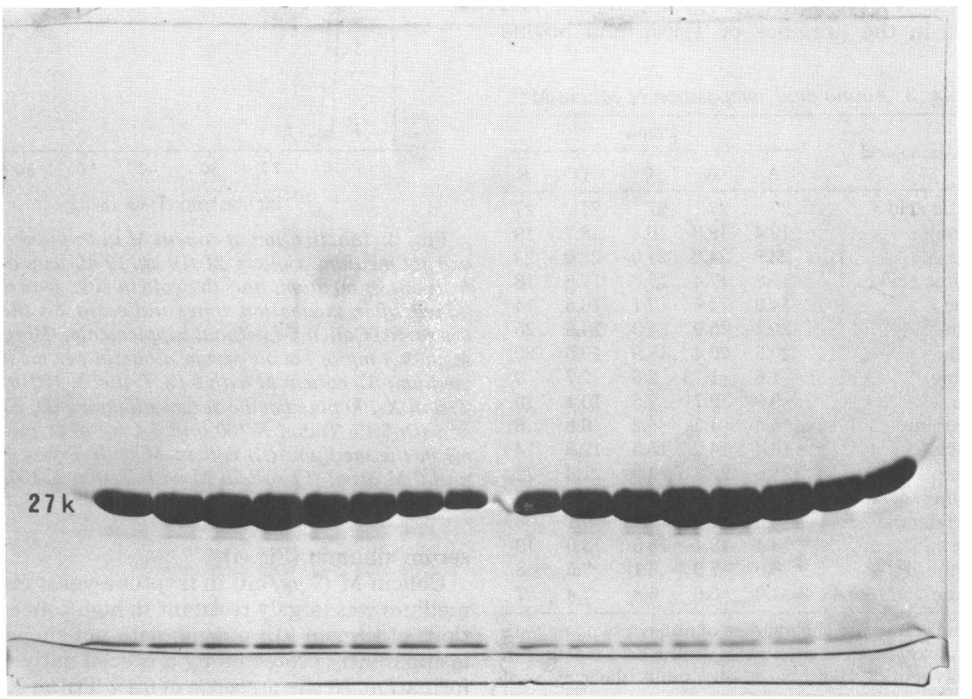

FIG. 2. *Electrophoresis of colicin M samples on polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate. The eight samples to the left were taken from fractions 353 through 346 (in this succession) of the carboxymethyl cellulose chromatography without the presence of Triton X-100 during the isolation procedure. The samples to the right were taken from fractions 370 through 363 of the preparation with Triton X-100 (see Fig. 1).*

served on the chromatogram of the amino acid analysis (see Discussion). The amino acid composition (Table 3) demonstrates the identity of the two colicin M preparations obtained after chromatography in the presence and absence of Triton X-100. Otherwise it showed no peculiarities except the content of three cysteine residues (see Discussion).

Stability of colicin M. Colicin M remained fully active over a period of 1 year in concentrations of at least 1 mg/ml. Shaking caused irreversible aggregation and precipitation. Colicin M was also rapidly denatured when diluted. Within 12 min all of the activity was lost (Fig. 3). Colicin M could be partly stabilized with bovine serum albumin; nonionic detergents like Tween 80, Brij 35, and Triton X-100 were more effective. Triton (0.1%), added to the medium before the colicin, prevented inactivation completely. None of these substances could renature inactivated colicin.

We obtained similar data when colicin M was added to growing cultures of *E. coli* IR20. Using a colicin concentration which caused a 50% decrease of the number of colonies without stabilization (Fig. 4), we found 0.1% of the colonies in the presence of bovine serum albumin (Fig. 4B), 0.03% in the presence of Triton X-100, and 0.008% in the presence of Triton and bovine serum albumin (Fig. 4).

TABLE 3. *Amino acid composition of colicin M*[a]

Amino acid	Prepn				
	A	B	C	D	E
Aspartic acid	27	27	27	27	27
Threonine	19.4	19.3	19.3	18.7	19
Serine	23.9	24.0	21.0	23.0	23
Glutamic acid	17.8	17.4	17.6	17.8	18
Proline	14.0	14.6	12.1	16.5	14
Glycine	25.2	25.9	24.3	24.6	25
Alanine	20.2	20.4	18.9	19.5	20
Cysteine	1.8	1.73	2.6	2.7	3
Valine	9.9	9.7	12.5	10.1	10
Methionine	5.3	4.7	6.2	6.8	6
Isoleucine	13.4	14.2	15.5	12.8	14
Leucine	21.6	22.3	22.9	21.3	22
Tyrosine	14.2	12.8	4.5[b]	3.7[b]	13
Phenylalanine	8.5	6.3	9.5	9.1	8
Lysine	13.4	13.4	13.5	13.0	13
Histidine	8.0	7.8	8.1	7.5	8
Arginine	7.0	6.6	6.8	7.4	7

[a] The values are the average of at least two analyses and are expressed as number of amino acid residues in one polypeptide chain based on a molecular weight of 27,000. Preparations A and C were without, and B and D were with, Triton X-100. (C, D) Samples were oxidized with performic acid. (E) Nearest integers of A through D. The values are relative to aspartic acid (27 residues). Tryptophan has not been determined.

[b] Tyrosine was partially destroyed during oxidation.

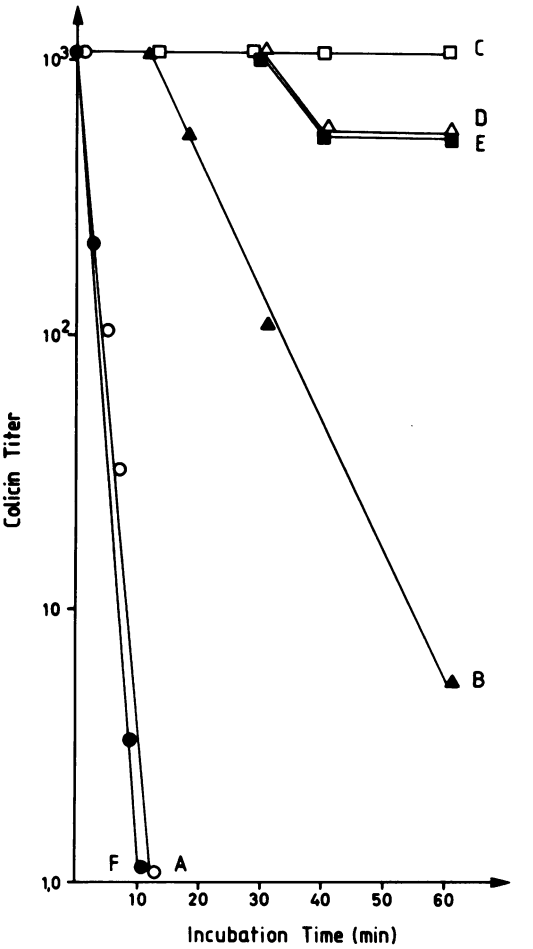

FIG. 3. *Inactivation of colicin M in tryptone-yeast extract medium. Colicin M (10 μg/10 μl) was added to 10 ml of medium, and the colicin titer was determined after incubation times indicated on the abscissa. (A) Colicin M without supplements; (B) colicin M with 4 mg of bovine serum albumin per ml in the medium; (C) colicin M with 0.1% Triton X-100 or with Triton X-100 plus bovine serum albumin; (D) colicin M with 0.1% Triton X-100 and 0.4 mg of trypsin per ml in the medium; (E) colicin M with Triton X-100 and 2 M urea; (F) colicin M with Triton X-100, 2 M urea, and trypsin (0.4 mg/ml).*

Colicin M (2 μg/ml) in tryptone-yeast extract medium was largely resistant to high concentrations of trypsin (0.4 mg/ml; data not shown). It is apparently protected by a special native conformation. In the presence of 0.1% Triton X-100, the colicin remained fully active for 30 min at 37°C (Fig. 3). However, rapid inactivation by trypsin occurred in the presence of urea and Triton X-100, indicating that the colicin became trypsin sensitive in the presence of 2 M urea

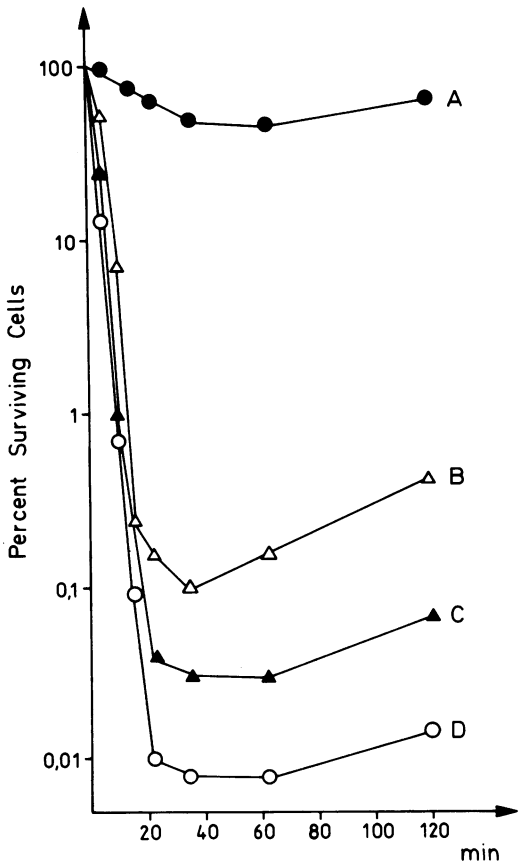

FIG. 4. *Surviving cells after treatment with colicin M. Colicin M (20 ng/20 μl) was added to a logarithmically growing culture of E. coli IR20 in 20 ml of tryptone-yeast extract medium. Samples were taken after the times indicated, and the viable cells were determined on tryptone-yeast agar plates. (A) Colicin M without additional supplements; (B) colicin M with bovine serum albumin (4 mg/ml) in the medium; (C) colicin M with 0.1% Triton X-100 in the medium; (D) colicin M with 0.1% Triton X-100 and bovine serum albumin (4 mg/ml) in the medium.*

(Fig. 3). The same concentration of urea alone denatured colicin M only slightly.

When the same amount of trypsin (0.4 mg/ml) was added together with colicin M (20 μg) to a 20-ml culture of *E. coli* IR20 in tryptone-yeast extract medium containing 0.1% Triton X-100, the cells continued to grow at the same rate as the control culture without colicin M. Addition of trypsin 2 min after colicin M produced the same result. However, when the cells were incubated with colicin M for 4 min before trypsin was added, the absorbance of the culture stayed constant for 3 min and then decreased within 6 min to 0.01. The absorbance of the cell culture decreased at the same rate to the final value obtained without trypsin. These results revealed two interesting phenomena. First, colicin M became trypsin sensitive in the presence of cells, in contrast to the stability of colicin M that was incubated with trypsin in the growth medium alone. Second, lysis of cells induced by colicin M could not be reversed by trypsin after 4 min of colicin action.

Rescue of colicin M-treated cells by treatment with sodium dodecyl sulfate. Sodium dodecyl sulfate (0.1%) immediately inactivated colicin M dissolved in tryptone-yeast extract medium. Added to a cell culture at different times after colicin M, it inactivated the cell-bound colicin to the same extent as 0.4 mg of trypsin per ml (data not shown). To study the time course of the killing of cells by colicin M in more detail, we used much lower colicin M concentrations (1 ng/ml). Sodium dodecyl sulfate added together with or up to 5 min after colicin M inhibited the killing action of colicin M completely (Fig. 5). When the detergent was added 10 min after colicin M, the number of viable cells dropped to 10%. There was still some protection after 15 min of colicin M action. After 25 min, most of the cells had already lysed so that no cells could be rescued by the detergent. We included in Fig. 5 also the time course of the absorbance change of the cell culture to show that the detergent did not inhibit growth and to demonstrate the correlation between viable cell counts and absorbance. The latter reveals the immediate events in the culture, whereas the former shows what happened to the cells after the colicin M and the detergent had been diluted out so that they were no longer active and the surviving cells had multiplied on plates overnight. Both measurements gave basically the same results.

Killing action of colicin M requires Ca^{2+}. Ca^{2+} (0.5 mM) increased the colicin M-induced lysis of cells. With colicin M concentrations which barely caused lysis, addition of Ca^{2+} strongly increased lysis even when added 15 min after colicin M (data not shown). If Ca^{2+} is essential, ethylene glycol-bis(β-aminoethyl ether)-*N*-*N*′-tetraacetate (EGTA) should prevent colicin action. In fact, 0.2 mM EGTA was sufficient to prevent the turbidity decrease of the colicin-treated culture (Fig. 6). Lower concentrations of EGTA (e.g., 0.1 mM) inhibited colicin M only partially. This effect was completely reversible. When 0.2 mM $CaCl_2$ was added back to cells treated with colicin M and EGTA, a decrease in optical density occurred similar to that in the culture which had contained only colicin M. EGTA did not prevent colicin M binding to cells. When colicin M was added to sensitive cells 5 min after EGTA and

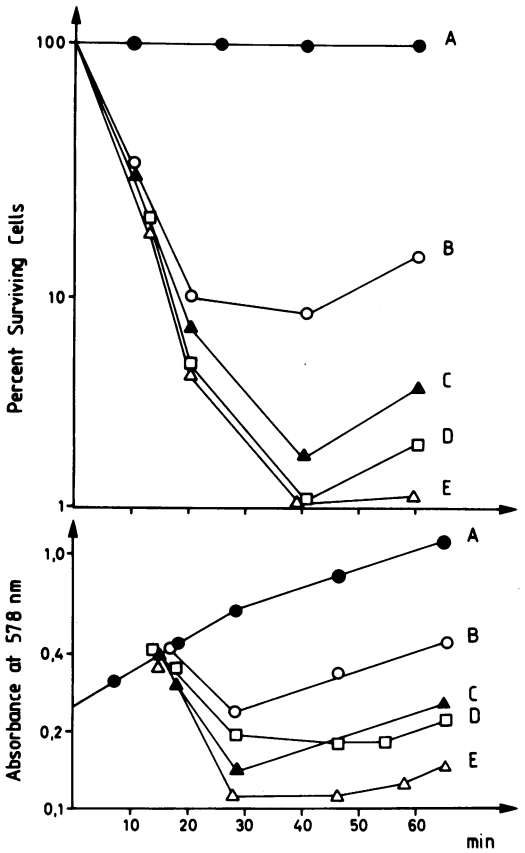

FIG. 5. *Inhibition of the colicin M-induced cell killing and lysis by sodium dodecyl sulfate. Colicin M (20 ng/20 μl) together with bovine serum albumin (4 mg/ml final concentration) was added at zero time to a logarithmically growing culture of E. coli IR20 in 20 ml of tryptone-yeast medium. Samples were taken at the times indicated, and the viable cells were determined on tryptone-yeast extract plates. Sodium dodecyl sulfate (final concentration, 0.1%) was added (A) at time zero or at 2, 5, (B) 10, (C) 15, or (E) 25 min after colicin M. (D) Colicin M without sodium dodecyl sulfate in the medium.*

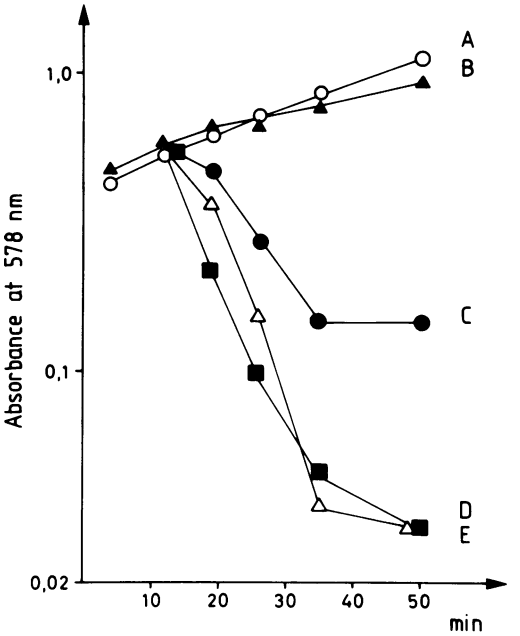

FIG. 6. *Inhibition of the colicin M-induced cell lysis by EGTA. Colicin M (10 ng/10 μl) was added at zero time to a logarithmically growing culture of E. coli IR20 in 20 ml of tryptone-yeast extract medium supplemented with Triton X-100 (0.1%). Growth was followed by measuring the absorbance of the culture at 578 nm. (A) Growth without additions; (B) 0.2 mM EGTA added 55 min before colicin M; (C) 0.1 mM EGTA added 55 min before colicin M; (D) 0.2 mM EGTA added 55 min before and 0.2 mM $CaCl_2$ or $MnSO_4$ added 7 min after colicin M. When $CaCl_2$ was replaced by 0.2 mM $MgSO_4$, a curve identical to that in (B) was obtained. (E) Control culture with colicin M without further additions.*

allowed to adsorb for another 5 min, the culture showed normal lysis after washing by centrifugation and suspension in prewarmed medium containing 0.2 mM $CaCl_2$. Mn^{2+} had a similar effect, but Fe^{3+} and Mg^{2+} ions had no influence on colicin M action (data not shown).

Addition of 0.2 mM EDTA reduced the growth rate considerably, in contrast to EGTA, which did not affect the growth rate. Parallel experiments with EDTA were therefore meaningless since colicin M caused lysis only on growing cells. Further experiments were performed to determine the amount of time that colicin M had to act on cells before added EGTA was unable to prevent lysis. Incubation of cells with colicin M (1.3 ng/ml) could be extended up to 15 min, after which addition of 0.2 mM EGTA still rescued the cells. In one example (Fig. 7), EGTA was added 10 min after colicin M. This result compares with the outcome of the rescue experiments with sodium dodecyl sulfate and shows that colicin M had to act on cells continuously until lysis set in (under these conditions, after 20 min).

Since EGTA prevented lysis and colicin M stayed cell bound, it was of interest to determine whether colicin M became, under the influence of EGTA, susceptible to degradation by trypsin. EGTA was added to growing cells 30 min before colicin M was supplied. Then trypsin (0.25 mg/ml) was added to one culture after 9 min and was omitted from the control culture. Without trypsin, cells lysed after $CaCl_2$ had been added (Fig. 7). Lysis was largely prevented in the culture supplied with trypsin. When the cells were not incubated with EGTA, the percentage of

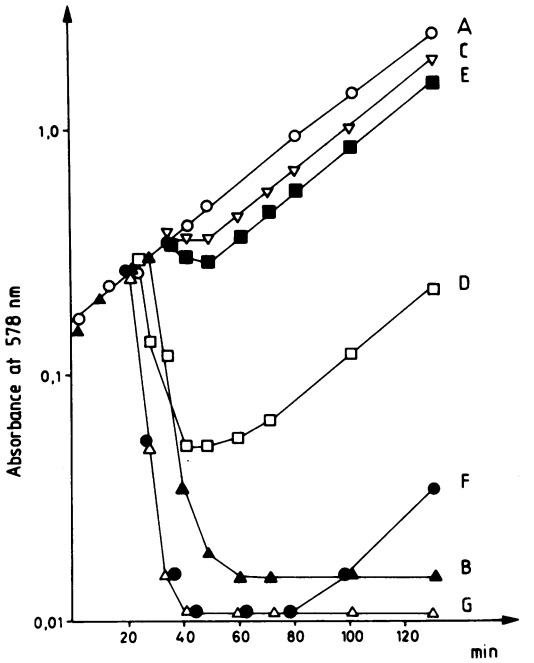

FIG. 7. *Restoration of the trypsin sensitivity of colicin M by treatment with EGTA. Colicin M (20 ng/20 μl) was added at zero time to 30 ml of a logarithmically growing culture of E. coli IR20. (A) 0.2 mM EGTA added 10 min after colicin M; (B) EGTA added 30 min before and 0.2 mM $CaCl_2$ added 20 min after colicin M; (C) EGTA added 30 min before, trypsin (250 μg/ml) added 9 min after, and $CaCl_2$ added 20 min after colicin M; (D) as in (C) but without EGTA; (E) as in (C) but EGTA added 10 min after colicin M; (F) colicin M without further supplementations; (G) $CaCl_2$ added after colicin M.*

cells rescued by trypsin was much lower. When EGTA was added shortly after trypsin, the action of colicin was also inhibited. Additional control cultures run in parallel demonstrated the action of colicin M alone and enhanced lysis induced by additional $CaCl_2$ (0.5 mM) in the growth medium. With low concentration of colicin M, we regularly observed the partial recovery of cells after prolonged incubation which was prevented in the presence of $CaCl_2$. This experiment clearly showed that colicin M remaining bound to the cells became again accessible to trypsin degradation under the action of EGTA.

Number of colicin M molecules needed to kill a single cell. The bacterial action of the different colicins is consistent with single-hit kinetics (15, 16), meaning that each colicin molecule has a definite probability of killing a sensitive bacterium. However, the average number of bound molecules for one killing event was in all cases above 10 (15, 16). We obtained similar data with colicin M. Following the idealized Poisson equation $N/N_o = e^{-m}$, where N/N_o corresponds to the fraction of the original cell population that remained viable, we found that a single lethal unit m corresponded to 10 molecules of added colicin M. One milligram of colicin M consisted of 2×10^{15} lethal units. We did not determine the number of adsorbed molecules per cell because radioactively labeled colicin M was also bound to cells which did not contain measurable quantities of receptor protein (*fhuA* = *tonA* mutants). The mutants remained completely unaffected by the colicin. The important finding, however, is that an extremely low concentration of colicin M killed the cells.

DISCUSSION

The strain we used in this study produced enough colicin M upon treatment with mitomycin C to allow isolation of apparently pure colicin M. The determination of the molecular weight on Sephadex G-100 of active colicin M, even from crude extracts (2), yielded the same value as electrophoresis of the pure, denatured colicin on polyacrylamide gels in the presence of sodium dodecyl sulfate and under reducing conditions. This shows that no immunity protein was associated with colicin M, as is the case with some other colicins (12, 19). This finding also demonstrates that the active molecule has a smaller molecular weight than the colicins so far characterized (12) and that no oligomers of two or more polypeptides occur. Concerning the amino acid composition, it is notable that the polypeptide contains three cysteine residues, which contradicts the general rule that excreted proteins usually lack cysteine.

Colicin M is more strongly associated with cells than is usually observed with colicins. It is also extremely unstable in dilute solutions, and previous preparations contained phospholipids, particularly phosphatidylethanolamine (2). These findings can be reconciled by assuming that colicin M contains hydrophobic amino acids at the surface which destabilize the molecule in aqueous solution. At a high protein concentration or, in the cell membrane, the hydrophobic areas are shielded by associations with proteins and phospholipids. The finding that Triton X-100 preserved the activity of colicin M supports this explanation and provides the condition for functional studies.

It is interesting that active colicin M in solution was largely resistant to trypsin and that it was degraded when bound to the cells. We assume that colicin M changes the conformation when adsorbed to the receptor and that this structural change causes the susceptibility to trypsin.

We have previously argued that colicin M

stays bound to the cell surface receptor in unenergized cells and in *tonB* mutants, since under these conditions it prevents binding of phage T5 and remains sensitive to trypsin (1). Here we show that growing cells were unaffected by colicin M when trypsin was added up to 4 min after colicin M. However, after 6 min cells could no longer be rescued by trypsin. It was necessary that colicin M act on cells continuously until they started to lyse, since EGTA still prevented cell death when added immediately before the onset of lysis (under the conditions used, after 20 min). We therefore conclude that after 4 min colicin M had become inaccessible to trypsin because the colicin had been translocated from the surface toward the cell interior. Denaturation of colicin M by sodium dodecyl sulfate followed the same time dependence. Colicin M was sensitive for 5 min after it had been added to cells and was stable thereafter. The resistance to trypsin could also have been explained by a conformational change of the colicin molecule, but this is unlikely to be the cause for the resistance to sodium dodecyl sulfate. Both trypsin and sodium dodecyl sulfate, even at much higher concentrations than those used in this study, do not reduce the viability and the growth rate of *E. coli* K-12 cells, since the outer membrane is impermeable to both agents. Therefore, the resistance of colicin M observed after about 5 min can be attributed to the uptake of colicin M during this time so that it is no longer exposed at the cell surface.

The inhibition of cell lysis by EGTA and its reversal by Ca^{2+} or Mn^{2+} showed a requirement for these divalent cations in the action of colicin M. Interestingly, Mg^{2+} was without effect. The experiments with EGTA also revealed that colicin M became trypsin sensitive again at a time (9 min) after which trypsin had no effect in the absence of EGTA. We take the restoration of the colicin sensitivity to trypsin as evidence that the colicin does not have to penetrate far into the cell to cause lysis. EGTA, unlike EDTA, did not reduce the growth rate of the cells at the concentrations used, so it is unlikely to penetrate far into the cell envelope either. After entering the cytoplasm, or merely penetrating into the cytoplasmic membrane, the colicin would probably not become trypsin sensitive upon treatment of cells with EGTA. This conclusion is supported by the following findings. Colicin M does not primarily reduce the rate of protein, DNA, and RNA synthesis (2), so it is not one of the colicins which dissipate the membrane potential (13). Treatment with colicin M leads to the inhibition of murein synthesis and the promotion of murein hydrolysis (K. Schaller, R. Dreher, and V. Braun, unpublished data). The enzyme system in question is likely to be located at the surface of the cytoplasmic membrane in proximity to the murein. In addition, we have isolated a temperature-sensitive, colicin M-tolerant mutant in which colicin M becomes trypsin sensitive at the nonpermissive temperature. This observation parallels that with EGTA and makes it unlikely that EGTA increases the permeability of the outer membrane for trypsin. We therefore favor the interpretation that the removal of an essential divalent cation, Ca^{2+} (or Mn^{2+}), inhibits colicin M action at its target site and causes a translocation of the colicin molecule toward the cell surface so that it became again accessible to trypsin. This aspect will be studied further.

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