Effects of Colicin K on a Mutant of Escherichia coli Deficient in Ca$^{2+}$,Mg$^{2+}$-activated Adenosine Triphosphatase

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SUMMARY

The effects of the bactericidal protein colicin K have been compared on a normal strain of Escherichia coli and a derivative, uncA, that is defective in Ca$^{2+}$,Mg$^{2+}$-activated ATPase activity. The syntheses of DNA, RNA, and proteins, which are completely arrested by colicin K in the normal E. coli cells, are only reduced by 30 to 50% in the ATPase-deficient cells. Intracellular ATP levels, which are reduced in the normal cells, are substantially increased by colicin K in the uncA mutant. The active transport of glutamine, which is coupled to phosphate bond energy, is drastically inhibited in both strains as is the transport of proline, a transport system that is coupled to an energized membrane state. These results provide the basis for the proposal that the primary action of colicin K is to de-energize the cytoplasmic membrane systems that are essential for a variety of active transport processes in E. coli. In cells having ATPase activity, ATP levels are diminished by the action of ATPase attempting to re-energize the membrane. This reduction in ATP levels in turn leads to an arrest of macromolecular biosyntheses. These secondary effects of colicin K on ATP levels and on synthetic processes are not present in uncA cells because they lack ATPase activity.

Colicin K is one of a class of protein antibiotics that bind to specific receptors in the outer membrane of Escherichia coli cells and affect various metabolic functions (for review, see Ref. 1). After binding one of these bactericidal proteins, sensitive cells pass through two definable stages (2-5). In Stage I no detectable physiological damage occurs and cell viability can be restored by a treatment with a proteolytic enzyme such as trypsin. In Stage II physiological damage becomes manifest and the cells can no longer be rescued by trypsin. The transition from Stage I to Stage II follows first order kinetics (2) and appears to require energy since it can be inhibited by cyanide and uncoupling agents (6-9). In the case of colicin K the transition has been shown to be sensitive to changes in the fluid properties of the E. coli envelope: Arrhenius plots for colicin K killing are biphasic and the temperature of discontinuity can be varied by substituting different unsaturated fatty acids in the membrane phospholipids (9).

The transition of cells treated with colicin K to Stage II brings about a number of alterations, some of which indicate damage to the cytoplasmic membrane. There is an immediate and simultaneous arrest of DNA, RNA, and protein synthesis (10); a decrease of intracellular ATP levels (11); and an inhibition of the active transport of β-D-galactosides (11), several amino acids (12, 13), and potassium (14), magnesium (15), and rubidium (14) ions. The cells eventually suffer a net loss of potassium (3, 12, 14) and magnesium (15) ions. Colicin K also inhibits the uptake of proline by membrane vesicles prepared from E. coli cells (16). Yet there are no gross changes in the permeability properties of the membrane since the facilitated diffusion of β-D-galactosides and the accumulation of α-methylglucoside remain nearly normal (11). Since most of the active transport systems affected by colicin K appear to be coupled to respiration or to some high energy state of the membrane it has been suggested (1) that the primary target of colicin K is the system that, within the cytoplasmic membrane of E. coli, couples electron transport to these active transport systems. How action on this target could also account for the drop in ATP levels and the arrest of macromolecular syntheses has not been clear.

Recently, Berger (16) reported the existence in E. coli of a coupling mechanism that is dependent on ATP or some form of phosphate bond energy and is utilized for the active transport of glutamine. The transport of glutamine, unlike that of proline (16, 17), is sensitive to arsenate and requires an active Ca$^{2+}$, Mg$^{2+}$-activated ATPase when a respiratory substrate such as D-lactate is used as energy source. Berger's (16) report prompted us to compare the effects of colicin K on the glutamine and proline transport systems of a normal E. coli strain and of a mutant (uncA) that lacks Ca$^{2+}$,Mg$^{2+}$-activated ATPase activity (18). We have found that colicin K inhibits both transport systems in the uncA mutant, even though in this mutant ATP levels do not decrease as a result of colicin K treatment. During the course of these studies we have also found that in the uncA mutant colicin K allows continued macromolecular syntheses at a substantial rate. The implication of these findings for the mode of action of colicin K will be discussed.

EXPERIMENTAL PROCEDURE

Materials—Trypsin, soybean trypsin inhibitor, and DNase I were purchased from the Worthington Biochemical Corp. ATP, dATP, dCTP, dTTP, and firefly lantern extract were ob-

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6138
strain and G6 metE was obtained following mutagenesis with stock collection No. 6 (Hfr, hisA323)) is the parental strain used shortened to 5 min.

Kobayashi and Anraku (21) showed less than 10% of the ATPase activity of G6, as assayed by own at 37° in Ozeki medium base (23) SuDDlemented with ous ilv mutant of G6 poZA1 by cotransduction of Uric- with ilv+ the method of De Lucia and Cairns (22).

Brunswick gyratory shaking water baths until the ce'll density from N144.

ml) to a cell density of 60 Klett units. The cultures (20 ml in G6 uncA was constructed from G6 ilv by Pl bacteriophage cotransduction (20) A membrane fraction prepared from G6 uncA by the method of Kobayashi and Anraku (21) showed less than 10% of the ATPase activity of the parent G6 strain. G6 poAI cells exhibited less than 2% of the DNA polymerase I activity of G6, as assayed by the method of De Lucia and Oders (22).

Media and Growth of Bacteria—Bacterial cultures were routinely grown at 37° in Ozeki medium base (23) supplemented with glucose (0.4%, w/v), thiamine (0.5 μg per ml) and histidine (100 μg per ml) to a cell density of 60 Klett units. The cultures (20 ml in 300-ml flasks) were then shifted to 27° and incubated in New Brunswick gyratory shaking water baths until the cell density reached 80 Klett units (1 Klett unit = 8 X 10^8 cells per ml). Viability assays and trypsin treatment were carried out as previously described (2) except that the incubation period with trypsin was shortened to 5 min.

Colicin K Preparation—Highly purified colicin K was prepared as previously described (9). The number of killing units in the colicin preparation was calculated from the equation S/Sb = e^−μt, where μ is the average number of killing units per cell and S/Sb in the survival ratio. Killing units of colicin K per cell, where expressed, were determined from viability measurements made 10 min after addition of colicin.

Uptake of Proline and Glutamine—Cells (5 X 10^8 per ml) were incubated with shaking at 27° in complete medium containing 100 μg of chloramphenicol per ml for 10 min. Aliquots (0.2 ml) were then transferred to tubes containing 20 μl of L-[U-14C]proline (10.6 μCi per μmole per ml) or 20 μl of L-[U-14C]glutamine (10.6 μCi per μmole per ml). Incubations were terminated by dilution with 2 ml of Ozeki medium base at room temperature followed by rapid filtration on nitrocellulose filters (pore size 0.45 μm; Matheson-Higgins, Inc., Woburn, Mass.). After washing with 4 ml of Ozeki medium base, the filters were glued to planchets and radioactivity was measured in a Nuclear-Chicago gas flow counter. Dilution, filtration, and washing were completed in less than 15 s.

DNA, RNA, and Protein Synthesis—Rates of DNA or RNA synthesis were determined by transferring 0.5-ml aliquots of cells (5 X 10^8 per ml) to tubes at 27° containing 50 μl of L-[2-14C]thymidine (5.5 μCi per μmole per ml) or 100 μl of L-[2-14C]uracil (40 μCi per μmole per ml), respectively. The incubations were terminated after 3 min by the addition of 5 ml of cold 10% (w/v) trichloroacetic acid. The acid-insoluble precipitates were collected on filters (pore size 0.6 μm; Schleicher and Schuell, Keene, N. H.; presoaked in N,N,N′-tri-n-butyl orthophosphoric acid, then with cold 0.1 N HCl; dried; and counted in a Beckman liquid scintillation spectrometer.

When L-[U-14C]glucose was used as precursor to measure the effects of DNA, RNA, and protein synthesis, the following procedure was employed. Cells were harvested at room temperature, washed once with Ozeki medium base, and then resuspended in Ozeki medium base supplemented with histidine, thiamine, and glucose (0.04%, w/v). The washed cells were incubated with shaking for 10 min prior to addition of colicin. At intervals 5-ml aliquots were transferred to 25-ml Erlenmeyer flasks containing trypsin (2.5 mg) and L-[U-14C]glucose (0.5 μCi). After 5 min at 27° with shaking, soybean trypsin inhibitor (2.5 mg) was added and the incubation was continued for an additional 10 min. Incubations were terminated with the addition of 5 ml of cold 20% (w/v) trichloroacetic acid. The lipids and nucleic acids were fractionated from the precipitates by a procedure described by Nomura and Maeda (24). The remaining trypsin-insoluble residue, comprising the protein fraction, was dissolved in 0.2 N NaOH.

RESULTS

Effects of Colicin K on Glutamine and Proline Transport—Colicin K is known to inhibit the active transport of several amino acids including proline (12, 13). We wished to compare the action of the colicin on proline transport with its effects on glutamine transport since these two transport systems appear to be coupled to different forms of energy (16). In view of the fact that the Ca^2+,Mg^2+ -activated ATPase has been implicated in energization of active transport systems in intact cells of E. coli (16, 25-29), we employed two strains, G6 and its derivative G6 uncA, which is deficient in ATPase activity. It is apparent from the results presented in Fig. 1 that for both G6 and G6 uncA cells treatment with colicin K greatly diminished the ability to transport glutamine as well as proline.

In the experiments presented in Fig. 2 the inhibition of glutamine and proline transport was followed from the time of addition of colicin K. With G6 cells (Fig. 2A) both glutamine and proline transport activities declined exponentially with time of colicin treatment. Their decline closely paralleled the rate at which the colicin-treated cells underwent the transition from Stage 1 of colicin action (cells resuscuable by trypsin) to Stage II (no trypsin rescue). These results are similar to those previously obtained for β-β-galactoside transport (2) and suggest that in Stage 1 of colicin K action the cells retain fully the ability to...
transport β-galactosides, proline, and glutamine; upon the transition to Stage II these transport activities are lost simultaneously or nearly so.

The results with the mutant cells deficient in ATPase activity are shown in Fig. 2B. Both proline and glutamine transport activities were strongly inhibited. Proline transport again declined at a rate commensurate with the loss of trypsin rescuability, whereas the inhibition of glutamine transport occurred with a certain delay and was less complete, the residual rate of transport depending on the concentration of colicin K used.

These experiments show that colicin K inhibits glutamine transport, a cellular function presumed to be dependent on ATP or at least on phosphate bond energy (16), almost as effectively as it does proline transport and other transport systems that are energized by electron transport (17).

Effects of Colicin K on ATP Levels—Since glutamine transport is supposed to have a direct requirement for ATP (16) and because colicin K is known to lower ATP levels (11), the inhibition of glutamine transport by colicin K might be an indirect effect of reduced ATP levels. That this is not the case became apparent when we measured the effects of colicin K on ATP levels in G6 and G6 uncA (Table I). Addition of colicin to G6 cells grown on glucose caused ATP levels to drop rapidly to about 70% of normal and then to remain nearly constant. If succinate instead of glucose was the carbon source, ATP levels dropped to 50% of normal (data not presented). In the ATPase-deficient cells of G6 uncA grown on glucose, however, treatment with colicin K increased ATP to over twice its normal level. Comparable rises in ATP levels were observed following colicin K treatment of G6 cells pretreated with the ATPase inhibitor N,N'-dicyclohexylcarbodiimide, a finding similar to that reported by Feingold for colicin E1 (30).

Effects of Colicin K on Macromolecular Syntheses in Presence and Absence of ATPase Activity—The effects of colicin K treatment on the rates of RNA and DNA synthesis in G6 and G6 uncA cells are shown in Figs. 3 and 4. In cells of G6 (Figs. 3A and 4A) both the rate of RNA synthesis and the rate of DNA synthesis decreased exponentially with time of colicin treatment. The fractional residual levels of these syntheses at any given time approximated the fraction of colicin-treated cells that could still be rescued by trypsin treatment.

A very different picture emerged from similar experiments on G6 uncA cells. As shown in Figs. 3B and 4B, the rates of RNA and DNA synthesis remained relatively high after colicin K addi-

Fig. 2. Effect of colicin K on the uptake of glutamine and proline and on cell survival following trypsin treatment. Immediately before and at the times indicated following addition of colicin K to cells shaken at 27⁰, samples were removed for trypsin treatment and for assay of amino acid uptake as described under "Experimental Procedure." In each sample amino acid uptake was measured for 1 min. Killing units per cell were 3 (A) and 4 (B).

Fig. 3. Effect of colicin K on the rate of RNA synthesis and on cell survival following trypsin treatment. Immediately before and at the times indicated following addition of colicin K to cells shaken at 27⁰, samples were removed for trypsin treatment and for measurement of the rate of RNA synthesis by the incorporation of [3H]uracil. The procedures are described under "Experimental Procedure." Killing units per cell were 7 (A) and 5 (B).
The present study of colicin K action on a mutant of *E. coli* that lacks Ca$^{++}$, Mg$^{++}$-activated ATPase activity has enabled us to dissociate the effects of this colicin on cellular functions. The inhibition of DNA, RNA, and protein synthesis that customarily follows colicin K action is greatly decreased in ATPase-deficient
The synthesis of the macromolecules were measured by the incorporation of label from \( \nu\{U-^{14}C\} \)glucose. The procedures are described under "Experimental Procedure." Killing units per cell were 5 (A) and 4 (B).

**TABLE II**

**DNA synthesis in tolucenized preparations of colicin \( K \)-treated cells**

The procedures were based on the methods described by Moses and Richardson (33). Aliquots (5 ml) were removed from cultures immediately before and 10 min following addition of colicin \( K \) (5 killing units per cell for G6 and 3 for G6 \( \text{uncA} \)) and centrifuged at room temperature. The cells were resuspended in 1 ml of 0.05 M potassium phosphate buffer (pH 7.4) containing 1% (v/v) toluene emulsified by brief sonication. The mixtures were shaken vigorously for 5 min at 37°C and then held on ice. DNA synthesis in the toluenized-treated cells was measured by the incorporation of \( \text{[methyl-3H]}\)TMP into acid-insoluble material. The complete reaction mixture (0.3 ml) contained 70 mM potassium phosphate buffer (pH 7.4), 33 mM MgCl\(_2\), 1.3 mM ATP, 33 \( \mu \)M dATP, dCTP, \( \text{[methyl-3H]}\)TTP (100 \( \mu \)Ci per mole), and 0.05 ml of tolucenized cells. \( N\)-Ethylmaleimide (NEM; 1.5 mM) was present where indicated. After incubation at 37°C for 30 min the reaction was stopped by the addition of 3 ml of cold 10% (w/v) trichloroacetic acid containing 0.1 M PP.

The precipitates were collected on filters (pore size 0.6 mm (Schröler and Schuell, Keene, N.H.), presoaked in 70 mM potassium phosphate buffer (pH 7.4) containing TTP (100 \( \mu \)g per ml); washed with cold 10% (w/v) trichloroacetic acid-0.1 M PP; then with cold 0.01 N HCl; dried and counted in a Beckman liquid scintillation spectrometer.

| Reaction mixture | \( \text{G6} \) | \( \text{G6 } \text{uncA} \) |
|------------------|---------------|------------------|---------------|
| **Untreated**    | **Untreated** | **Untreated**    | **Untreated** |
| \( \text{ATP} \)  | 28            | 17               | 17            |
| \( \text{NEM} \)  | 2             | 2                | 2             |

The inability of colicin \( K \)-treated cells to retain amino acids and ions may lead to lowered rates of macromolecular synthesis (39). Another source of depressed synthesis may be a reduced efficiency of glucose utilization by colicin \( K \)-treated cells, in which pyruvate becomes the major excreted product of glucose catabolism (35).
Although G6 uncA cells are able to synthesize macromolecules at appreciable rates after colicin K treatment, the lack of ATPase activity does not affect the rate at which the cells are killed, at least as tested by plating on ordinary media. The inability of the colicin-treated cells to retain metabolites and ions may be the basis for this loss of viability. Unpublished experiments indicate that an uncA mutant can grow and divide in the presence of colicin K providing certain essential nutrients and ions provided at high concentrations in the growth medium.

REFERENCES

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