STIMULATION OF KANAMYCIN PHOSPHOTRANSFERASE SYNTHESIS IN *ESCHERICHIA COLI* BY 3',5'-CYCLIC AMP

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Studies on the mechanism of drug-resistance shown by resistant microorganisms isolated from patients were initiated in 1965 by Okamoto and Suzuki[1] who proved that strains carrying resistance (R) factor produce enzymes capable of inactivating chloramphenicol, streptomycin and kanamycin. *Escherichia coli* cells harboring R factors which confer on the host resistance to chloramphenicol synthesize constitutively an R factor-directed enzyme[2], chloramphenicol acetyl transferase (CAT), that inactivates this drug *via* acetylation[1,3,4]. Recently, Harwood and Smith[5] reported that CAT, and another R factor-directed enzyme, streptomycin adenylyltransferase[6], are subject to cAMP*-mediated, catabolite repression. Other aminoglycosidic antibiotics, such as kanamycin and paromamine, were reported to be inactivated mainly *via* phosphorylation of the OH at C-3 of the 6-glucosamine (kanamycin) or 2-glucosamine (paromamine) moiety[7,8]. The R factor responsible for these inactivations was transferred to *E. coli* K 12 from a drug-resistant *E. coli* isolated from patients and the resulting resistant strain was named *E. coli* K 12 ML-1629[7]. An enzyme preparation which phosphorylates kanamycin (as well as paromamine) was obtained from this strain[7,8]. In the present paper we will report our results which suggest that kanamycin phosphotransferase is also under cAMP-mediated, catabolite repression.

Methods

First culture: *E. coli* K 12 ML-1629 (T^-) cells were grown overnight at 37°C under reciprocal shaking in Sakaguchi flasks each containing 250 ml of the minimal salt medium (see below) supplemented with 10 μg/ml of thymidine and either 0.4 % (w/v) of glucose (Glucose-medium) or 0.4 % of glycerin (Glycerin-medium). The minimal salt medium contained in 4,000 ml; 52.5 g of K_2HPO_4, 22.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 5 g of $(NH_4)_2SO_4$, 2.25 g of Na-citrate and sufficient amount of water.

Second culture: From the first culture in glucose-medium, 25 ml was withdrawn and added to 225 ml of fresh glycerin-medium which contained, in addition, either kanamycin sulfate at a concentration of 77.8 μg/ml or kanamycin sulfate at the same concentration plus 5.5 mM cAMP. On the other hand, from the first culture in glycerin-medium, 50 ml was withdrawn and added to 200 ml of fresh glycerin-medium which contained, in addition, kanamycin sulfate at a concentration of 87.5 μg/ml. Second culture was conducted as the first culture until the optical density of the cultures at 600 mμ reached 0.7.

Preparation of enzyme solution[9]: All the procedures were performed at 5°C. After the second culture, cells were harvested by centrifugation at 12,700 *g* (max) for 10 minutes. From a 250 ml culture, generally 1.1~1.9 g of wet packed cells were obtained. Each cell pellet was washed by suspension and centrifugation in 15 ml×2 of TMK buffer which contained in 1,000 ml; 60 ml of 1 M KCl, 10 ml of 1 M $MgCl_2$, 6 ml of 1 M mercaptoethanol, 100 ml of 1 M Tris-HCl (pH 7.9) and sufficient amount of water. Washed cells were suspended in 4 volumes of TMK buffer and disrupted by passing through a French pressure cell (1,200 kg/cm^2). The disrupted cell suspension was centrifuged at 26,600 *g* (max) for 20 minutes and the supernatant was further centrifuged at 137,000 *g* (max) for 90 minutes. The supernatant was dialyzed against TMK buffer for 15 hours and the retentate was diluted with the same buffer to 10 mg proein/ml, which was used as enzyme solution. Protein concentration was determined by

* Abbreviation ; adenosine-3',5'-cyclic monophosphate

LOWRY's[10] method using bovine serum albumin as standard. Inactivation of kanamycin by each enzyme preparation was determined as follows: The assay mixture contained in a final volume of 0.50 ml; 0.25 ml of ATP solution (see below), 0.20 ml of enzyme solution, and 0.05 ml of kanamycin sulfate solution whose concentration was 7 mg/ml. The ATP solution contained in 25 ml; 5 ml of 1 M Tris-HCl (pH 7.9), 0.22 g of KCl, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.3 ml of 1 M mercaptoethanol, 2.4 g of ATP·2 Na and 0.65 g of $NaHCO_3$. Each assay mixture was incubated at 37°C for 3 hours and the enzyme was inactivated by heating the mixture in a boiling water bath for 3 minutes. Subsequently, a 50 μl aliquot was taken from the mixture and diluted with 950 μl of water. Runs were duplicated or triplicated (see Table 1) from the second culture through this stage. Antibiotic titers of the resulting solutions were determined by the paper disc method with *Bacillus subtilis* PCI 219 as an indicator. The titer of a blank run, which received heat-denatured enzyme at the beginning, was equal to 35 μg/ml of kanamycin sulfate and this titer is expressed as "0 % inactivation." Titration of each solution was conducted on 4 separate agar-plates and the results were averaged.

Table 1.

	Medium in which cells were grown (2 nd culture***)	Specific activity of kanamycin phosphotransferase in cell-free extracts (Rate of inactivation of kanamycin)	
Exp. A	(I) Glucose	37.0% 52.0 60.0	av. 49.7±11.7
	(II) Glucose-cAMP	90.6 92.4 94.5	av. 92.5± 1.9
	(III) Glycerin	91.0 85.2	av. 88.1± 8.4
	* Combination of cell-free extracts $\frac{1}{2}$ vol. of (I) $+\frac{1}{2}$ vol. of (II)	82.7 77.0 81.7	av. 80.5± 3.0
Exp. B	(I) Glucose	52.0% 60.0	av. 56.0± 4.0
	(II) Glycerin	91.0 85.2	av. 88.1± 8.4
	* Combination of cell-free extracts $\frac{1}{2}$ vol. of (I) $+\frac{1}{2}$ vol. of (II)	80.0 81.0	av. 80.5± 0.5
Exp. C	(I) Glucose	37.0% 52.0 60.0	av. 49.7±11.7
	** Cell-free extract of (I)+cAMP	38.0 47.0 58.0	av. 47.7±10.1

*, ** : see text. *** : see methods.

Results

E. coli K 12 ML-1629 (T^-) cells which are resistant to tetracycline, chloramphenicol and several aminoglycosidic antibiotics were grown in either glucose medium, glucose medium supplemented with cAMP or glycerin medium. In late exponential growth phase, cells were harvested and ruptured by passing through a French pressure cell and cell-free extracts were prepared. Specific activity of kanamycin phosphotransferase of these extracts was determined as described under Methods. As shown in Table 1, the enzyme activity was low in the cells grown in glucose medium while the activity was high in the cells grown in either glucose-cAMP medium or glycerin medium. Since the effect of combination of these enzyme preparations appeared almost additive in respect to the activity, it was unlikely that any inhibitor of the enzyme was produced in cells grown in glucose medium (*Table 1). Obviously, cAMP had no direct stimulation on the enzyme activity when added to a cell-free preparation (**Table 1). A separate experiment (results are not shown) revealed that the doubling time of this resistant *E. coli* strain in glucose medium, to which as much as 700 μg/ml of kanamycin sulfate was added, was not significantly influenced by the presence or absence of 5 mM cAMP. Consequently, the catabolite-repressed level of this enzyme in cells grown in glucose medium should be sufficient to inactivate the permeating amount of kanamycin.

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