NEW POLYENIC ANTIBIOTICS ACTIVE AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

VI. NON-LACTONIC POLYENE ANTIBIOTIC, ENACYLOXIN IIa, INHIBITS BINDING OF AMINOACYL-tRNA TO A SITE OF RIBOSOMES

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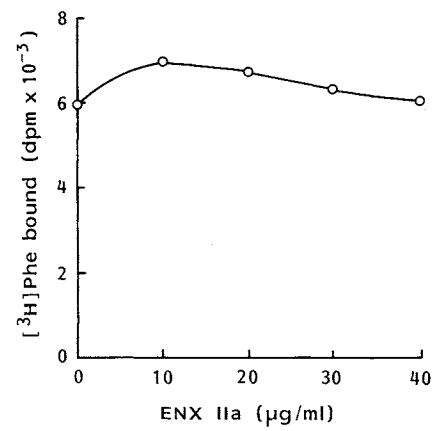
Enacyloxin (ENX) IIa is a polyenic antibiotic having a linear structure[1] and its antibiotic action is shown to be limited to Gram-positive and Gram-negative bacteria[2]. We have been interested in the mode of action of ENX IIa because it is very unique in its chemical structure. In the preceding paper[3], we described that ENX IIa markedly inhibited the incorporation of L-[^{3}H]phenylalanine into TCA-insoluble fractions of both growing cells and cell-free system (S-30 fraction) of *Escherichia coli*. From these results, the primary site of action of ENX IIa seems to be protein synthesis. In this paper, a more detailed mode of action of ENX IIa is described.

E. coli K-12 was used throughout this work. Cells were aerobically grown in nutrient broth at 30°C. At the logarithmic phase of growth, cells were harvested, centrifuged and washed twice with 0.01 M Tris-HCl, pH 7.8, then stocked at −20°C until use. Crude extract (S-30), magnesium chloride-washed ribosomes (free of elongation factor (EF)) and L-[^{3}H] or [^{14}C]phenylalanyl-tRNA were prepared as described by NIRENBERG[4], LUCAS-LENARD and LIPMANN[5], ZUBAY[6] and NISHIMURA *et al.*[7], respectively. EF-Tu prepared from *E. coli*[8] was kindly provided by Dr. S. EJIRI. L-Phenyl[2,3-^{3}H]alanine (1.7×10^6 MBq/mmol) and L-[*U*-^{14}C]phenylalanine (16.6 GBq/mmol) were obtained from Amersham International, England. L-[4,5-^{3}H]Leucine (1.5×10^6 MBq/mmol), L-[4,5-^{3}H]lysine (1.7×10^6 MBq/mmol), L-[^{3}H]arginine (3.7×10^5 MBq/mmol), L-[5-^{3}H]proline (6.7×10^5 MBq/mmol) and L-[^{3}H]threonine (7×10^5 MBq/mmol) were purchased from ICN Radiochemicals, U.S.A. ENX IIa was prepared as described previously[3]. Other chemicals were obtained from Wako Chemicals Co., Ltd., Osaka, Japan. Radioactivity trapped on the filter was counted by a liquid scintillation spectrometer (Aloka LSC-903) using the scintillation liquid of toluene-Nonion system[9]. ENX IIa was usually dissolved in 1% $NaHCO_3$, but it was dissolved in 95% methanol in the experiment shown in Table 2.

On the first step of this study, we determined if ENX IIa inhibits the aminoacyl-tRNA synthetase activity which catalyzes amino acid activation and formation of aminoacyl-tRNA. As shown in Fig. 1, ENX IIa had no inhibitory activity on aminoacyl-tRNA synthetase at the concentrations of 10~40 μg/ml although a small extent of stimulation was observed at the lower concentration.

NIRENBERG and LEDER[10] devised a rapid method of detecting the binding of aminoacyl-tRNA to washed ribosomes in the presence of various synthetic mRNAs. The reaction mixture consists of Tris-acetate, magnesium acetate, washed ribosomes, poly U and L-[^{3}H]phenylanyl-tRNA as indicated in Table 1. In this method, aminoacyl-tRNA bound to ribosomes with mRNA remained on the cellulose

Fig. 1. Effects of ENX IIa on the activation of amino acids and formation of aminoacyl-tRNA.



Assay of aminoacyl-tRNA synthetase (ARSase) activity was carried out according to NISHIMURA *et al.*[7]. Reaction mixture (100 μl) contains: 10 mM Tris-HCl, pH 7.5; 10 mM KCl; 2 mM ATP; 5 mM $(CH_3COO)_2Mg$; 2.6 A_{260} units of tRNA and 10 μl of ARSase. ENX IIa was added as indicated. Incubation continued for 20 minutes at 37°C, and each reaction was terminated by the addition of 5% TCA at final concentration. Reaction mixture was poured onto a glass fiber filter DP-70 (Toyo Roshi International, Japan) and washed with a mixture of ether-ethanol (1:1) to remove unbound L-[^{3}H]phenylalanine.

nitrate filter, while unbound aminoacyl-tRNA was washed out, although a small amount of unbound aminoacyl-tRNA in the absence of mRNA remained on the filter. We employed this method as a preliminary experiment to determine the effect of ENX IIa on the mRNA-dependent aminoacyl-tRNA binding to ribosomes. In this experiment, the radioactivity of aminoacyl-tRNA remaining on the filter in the absence of mRNA was subtracted as a blank from that in the experiment. As shown in Table 1, ENX IIa inhibited this binding reaction strongly, while tetracycline (TC) and erythromycin (EM), showed less inhibitory effects on this reaction.

Table 1. Effects of antibiotics on L-[^{3}H]phenylalanyl-tRNA binding to poly U-ribosome complex without cofactors (rapid method).

Antibiotics		[^{3}H]Phenylalanyl-tRNA bound to ribosomes	
		dpm/ 2 A_{260} units	Ratio
None		1,400	1.00
ENX IIa	5 μg/ml	394	0.28
	10 μg/ml	354	0.25
Tetracycline	50 μg/ml	1,092	0.78
Erythromycin	100 μg/ml	1,218	0.87

Standard reaction mixture (100 μl) contains: 0.1 M Tris-acetate, pH 7.2; 0.02 M $(CH_3COO)_2Mg$; 0.05 M KCl; 2.0 A_{260} units of ribosomes; 60 μg of poly U and 0.3 pmol of L-[^{3}H]phenylalanyl-tRNA. Antibiotics were added as indicated. After 20 minutes of incubation at 24°C, tube was placed in ice and the reaction was terminated with immediate dilution of ice-cooled 3 ml of 0.1 M Tris-acetate buffer, pH 7.2, containing 0.02 M $(CH_3COO)_2Mg$ and 0.05 M KCl. The diluted reaction mixture was immediately poured on a Millipore filter (HAWP; pore size 0.45 μm), which was washed with the same dilution buffer before use, and washed to remove unbound L-[^{3}H]phenylalanyl-tRNA.

The effect of EM was comparable to that of the reported experiment by MAO and WIEGAND[11]. This difference in the effects of EM and ENX IIa may be due to the difference in their chemical structures. In the bacterial cell-free system of protein biosynthesis, the complete ribosomal system for aminoacyl-tRNA binding reaction consisting of aminoacyl-tRNA, synthetic mRNA, ribosomes and cofactors (elongation factor Tu (EF-Tu) and GTP) should be used. Therefore, we employed this complete system for the binding of aminoacyl-tRNA-GTP-EF-Tu complex to A site of ribosomes and the effect of ENX IIa was examined. Since ENX IIa is more easily dissolved in methanol than 1% $NaHCO_3$ solution, we used 95% methanol for

Table 2. Effects of ENX IIa on L-[^{14}C]phenylalanyl-tRNA binding to ribosomes with cofactors (complete system).

		[^{14}C]Phenylalanyl-tRNA bound to ribosomes	
		pmol/ 2.6 A_{260} units	Ratio
None		1.10	1.00
ENX IIa	10 μg/ml	0.80	0.73
	20 μg/ml	0.65	0.59

Standard reaction mixture (100 μl) contains: 50 mM Tris-HCl, pH 7.5; 150 mM NH_4Cl; 8 mM $(CH_3COO)_2Mg$; 2 mM DTT; 2 mM GTP; 10 μg of poly U; 2.6 A_{260} units of ribosomes, 8 pmol of L-[^{14}C]phenylalanyl-tRNA and 85 pmol of EF-Tu. ENX IIa dissolved in 95% methanol was added as indicated and control experiments without ENX IIa were carried out with an addition of each corresponding amount of methanol as with ENX IIa. Incubation continued for 10 minutes at 37°C. Others were the same as in Table 1.

Table 3. Effects of ENX IIa on incorporation of various labeled amino acids in the presence of several synthetic mRNAs.

		Relative value of incorporation					
		Poly U as mRNA		Poly A as mRNA		Poly C as mRNA	
		[^{3}H]Phe	[^{3}H]Leu	[^{3}H]Lys	[^{3}H]Arg	[^{3}H]Pro	[^{3}H]Thr
None		100	2	100	4	100	15
ENX IIa	10 μg/ml	39	2	21	1	12	5
	50 μg/ml	7	2	9	1	5	5
	200 μg/ml	1	2	5	1	2	1

Reaction was carried out according to our preceding paper using S-30 fraction of *Escherichia coli*[3]. Each mRNA and labeled amino acid were added as the amounts of 60 μg and 1.8×10^4 Bq per tube, respectively. Polypeptides synthesized without ENX IIa per mg protein were as follows: 37 pmol for polyphenylalanine, 64 pmol for polylysine and 42 pmol for polyproline.

dissolving ENX IIa in the experiment shown in Table 2. As shown in Table 2, ENX IIa seemed to inhibit the binding of aminoacyl-tRNA-GTP-EF-Tu complex to A site of ribosomes significantly although the effect of ENX IIa is less than that shown in Table 1. The difference in inhibitory effects of ENX IIa with rapid and complete methods was not studied in detail because the mechanism of binding in the rapid method is not physiologically clear. Next, we examined the effect of ENX IIa on the incorporation of various ^{3}H-labeled amino acids into the TCA insoluble fraction in the presence of various synthetic mRNAs to find out whether ENX IIa has a miscoding effect like the aminoglycoside antibiotics. As shown in Table 3, ENX IIa had no appreciable miscoding effect.

In conclusion, ENX IIa seems to inhibit aminoacyl-tRNA binding to A site of ribosomes in the peptide chain elongation reaction in the protein synthesis of *E. coli*. This is the first report on the detailed mode of action of a polyenic antibiotic on the protein synthesis in *E. coli*.

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