IN VITRO INACTIVATION OF ASCITES RIBOSOMES BY COLICIN E 3

F. Turnowsky, J. Drews, F. Eich, G. Högenauer

SANDOZ Forschungsinstitut Ges.m.b.H.
A-1235 Vienna, Brunnerstraße 59 / Austria

Received March 12,1973

Summary: Colicin E 3 treatment of 80 S ribosomes from mouse ascites cells completely arrests in vitro protein synthesis. Isolated 40 S subunits are resistant to the colicin action while the larger subunit becomes inactivated after treatment with this protein. 40 S subunits derived from colicin E 3 treated 80 S ribosomes lose their ability to participate in polyphenylalanine synthesis. Colicin E 3 damaged 80 S ribosomes appear to be functional with regard to Met-$tRNA_f^{Met}$ binding while they fail to attach Phe-tRNA to the A-site. Thus, except for the susceptibility of their larger subunits to colicin, the inactivation mechanism of 80 S particles resembles the process which alters the bacterial ribosome.

Introduction: The bacteriocin colicin E 3 is a narrow spectrum antibiotic effective against certain E.coli strains, which differ from all other bacteria by carrying specific receptors on their surface. In bacteria, the in vivo mode of action of colicin E 3 involves specific cleavage of an RNA fragment off the small ribosomal subunits thus rendering them inactive in protein biosynthesis (1, 2). Colicin E 3 was also found to be active when added to a cell-free preribosomal supernatant or to isolated ribosomes from E.coli (3, 4). Recently it has been shown that ribosomes derived from bacterial strains which are unrelated to E.coli (5) are also sensitive to colicin E 3 in vitro. In this communication experiments are described which demonstrate that, in vitro, even mammalian ribosomes are inactivated by colicin E 3 and that a mechanism similar to the inactivation of bacterial ribosomes is involved.

Materials and Methods: E.coli CA 38 was kindly supplied by Dr. P. Fredericq, Krebs II ascites cells were a gift from Dr. I. Kerr. Poly-U and puromycin were purchased from Serva, Heidelberg, ApUpG was obtained from Boehringer, Mannheim, [^{35}S]Methionine (42 Ci/mMole) and [^{3}H]Phenylalanine (15.7 Ci/mMole) were products of the Radiochemical Centre, Amersham. The N-Hydroxy-succinimide ester of formic acid was synthesized by Dr. F.M. Unger.

Colicin E 3 was prepared from a culture of E.coli CA 38 according to Glick et al. (6). Procedures describing the growth and isolation of ascites cells have been published previously (7). The isolation of both tRNA and elongation factor EF-1, the separation of $tRNA_f^{Met}$ and $tRNA_m^{Met}$ as well as the aminoacylation of tRNAs have also been described (7). The procedure given by Felicetti and Lipman (8) was followed in order to isolate elongation factor EF-2 from ascites cells. Met-$tRNA_f^{Met}$ was chemically formylated with N-Hydroxysuccinimide ester of formic acid using the procedure of Gillam et al. (9). A postmitochondrial supernatant was prepared as described by Eggen and Shatkin (10). For the isolation of "run off" 80 S ribosomes the method of Mathews and Korner (11), for the preparation of subunits that of Blobel and Sabatini (12) was used.

Except for the binding reaction of initiator tRNA the ribosomes were twice washed with a high molar salt buffer. The solution of ribosomes in a buffer containing 200 mM NH_4Cl, 20 mM Tris.HCl pH 7.5, 5 mM magnesium acetate, and 1 mM dithioerythritol was mixed with an equal volume of 1 M KCl, 100 mM triethanolamine pH 7.5, 5 mM magnesium acetate, and 1 mM dithioerythritol. From this solution the ribosomes were collected by centrifugation.

Unless otherwise stated ribosomes or ribosomal subunits were incubated with colicin E 3 for 1 hour at 37°C at a concentration of 30 µg colicin E 3 per A_{260} unit of ribosomes. Controls were identically treated except that water was supplemented for the omitted colicin solution.

Results and Discussion: When a postmitochondrial supernatant from mouse ascites tumor cells is incubated with colicin E 3 it partially loses its ability to synthesize poly-U coded polyphenylalanine (Fig. 1). When 80 S ribosomes isolated from ascites cells are treated with colicin E 3 their poly-U directed polyphenylalanine synthesis catalyzed by EF-1 and EF-2 also ceases (Fig. 2). In this respect the E.coli and the mammalian ribosomes behave similarly. As shown in Fig. 3 and in Table 1, inactivation of 80 S ribosomes with colicin E 3 does not affect the binding of Met-$tRNA_f^{Met}$

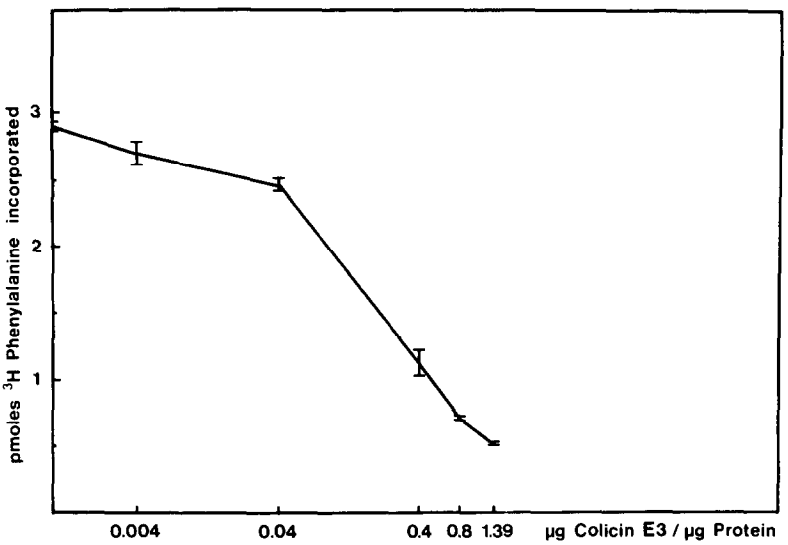

Fig. 1: Action of colicin E 3 on postmitochondrial supernatant. 0.69 A_{260} units of supernatant, containing 166 µg protein were incubated 60 minutes at 37° C with the amount of colicin E 3 indicated on the abscissa. The reaction mixture contained in 100 µl: 16 mM Tris.HCl pH 7.5, 75 mM KCl, 7 mM magnesium acetate, 7 mM ß-mercaptoethanol, 10 mM creatine phosphate, 1 mM ATP, 0.6 mM CTP, 0.1 mM GTP, 0.23 A_{260} units poly-U and 5 pmoles Phe-tRNA. Poly-phe synthesis was measured as described in Table 2.

(ascites) or f-Met-$tRNA_f^{Met}$ (ascites) to the ribosomal P-site of such ribosomes. P-site-bound formyl-methionine can be released by puromycin (Table 1). However, EF-1 catalyzed attachment of Phe-tRNA (ascites) to the ribosomal A-site is impaired (Fig. 4). Both colicin-treated 80 S- and E.coli-ribosomes (14) share these features.

Boon (15) observed that 30 S ribosomal particles from E.coli are resistant to the colicin E 3 induced RNA cleavage provided the larger ribosomal subunit is absent. Only the simultaneous presence of both ribosomal particles renders the small subunit sensitive to colicin E 3. We found a similar pattern in the mammalian system.

When 40 S subunits were incubated with colicin E 3 and subsequently centrifuged through a sucrose gradient, the reisolated particles, after being mixed with a twofold excess of 60 S subunits, yielded intact ribosomes

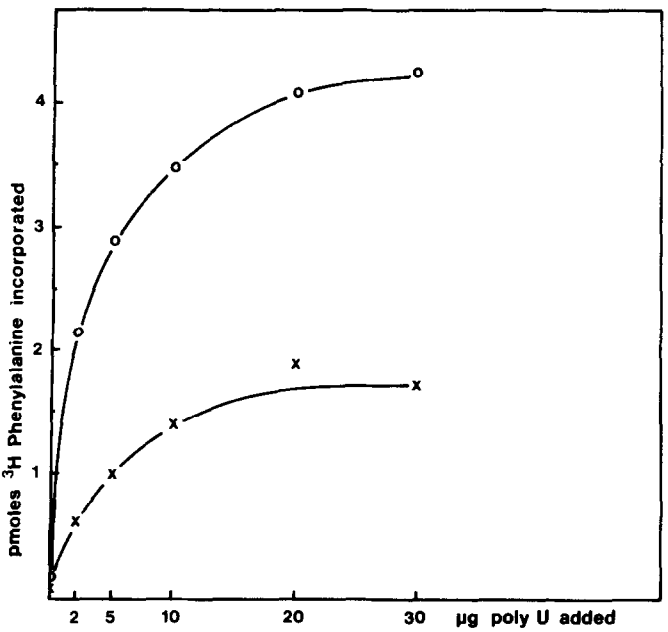

Fig. 2: Action of colicin E 3 on 80 S ribosomes. 100 µl incubation mixture contained: 1 A_{260} unit 80 S ribosomes, 11 mM Tris.HCl pH 7.5, 80 mM KCl, 10 mM NH_4Cl, 5 mM magnesium acetate, 6 mM ß-mercaptoethanol, 10 mM creatine phosphate, 10 µg creatine kinase, 1 mM ATP, 0.6 mM CTP, 0.1 mM GTP, 30 µg EF-1, 10 µg EF-2, 5.5 pmoles Phe-tRNA, and poly-U as indicated on the abscissa. Poly-phe synthesis was measured as described in Table 2. o-o control ribosomes, x-x colicin E 3 treated ribosomes.

---

which suffered only a small (5-20 %) loss of polyphenylalanine synthesizing capacity as compared to reassociated control particles (Table 2). This small decrease in poly-phe synthesis may be explained by an impurity of 60 S subunits in the 40 S preparation (data not shown) which makes a few 40 S subunits colicin sensitive.

When both small and large subunits, derived from colicin E 3 treated 80 S ribosomes are allowed to reassociate with subunits similarly isolated from normal ribosomes, both the large subunit and, to a lesser extent, the 40 S particle exhibited an impaired polyphenylalanine synthesizing capacity (Table 3).

When 60 S subunits, after incubation with colicin E 3 and centrifugation

Table 1

f-Met-tRNA binding to unwashed 80 S ribosomes and f-Met-puromycin release

	f-Met-$tRNA_f^{Met}$ bound (pmoles)	f-Met-puromycin released (pmoles)
Ribosomes + Colicin E 3	0.38	0.12
Control Ribosomes	0.34	0.14

f-Met-tRNA binding: 2 A_{260} units of colicin treated and of control ribosomes were incubated for 10 minutes at 30° C with 20 pmoles of f-Met-$tRNA_f^{Met}$ and 0.2 A_{260} units of ApUpG in a medium containing in 100 µl: 22 mM Tris.HCl, pH 7.5, 80 mM KCl, 20 mM NH_4Cl, 5.5 mM magnesium acetate, 2 mM dithioerythritol, and 0.5 mM GTP. The reaction mixtures were filtered on Millipore filters, washed, dried and counted.
f-Met-puromycin formation: Two reaction mixtures as described for f-Met-tRNA binding were mixed after a 10 minute incubation with 10 µl of a 10 mM puromycin solution and incubated for another 10 minutes. 1 ml of 100 mM Na-phosphate buffer, pH 8, and 2 ml of ethyl acetate were subsequently added. After shaking an aliquot of the organic phase was removed and counted (13).

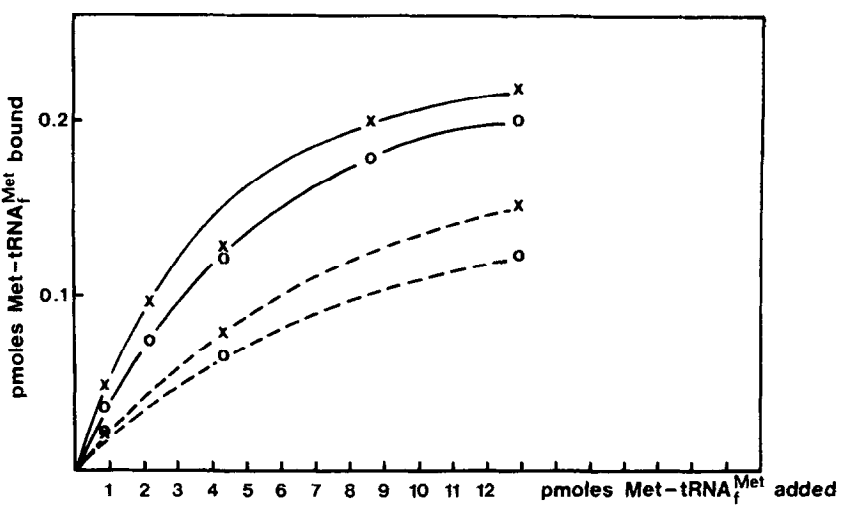

Fig. 3: Met-$tRNA_f^{Met}$ binding to 80 S ribosomes. 100 µl reaction mixture contained: 1.8 A_{260} units of unwashed 80 S ribosomes, 22 mM Tris.HCl pH 7.5, 80 mM KCl, 20 mM NH_4Cl, 5.5 mM magnesium acetate, 2 mM dithioerythritol, 0.5 mM GTP, 0.2 A_{260} units ApUpG, and Met-$tRNA_f^{Met}$ as indicated on the abscissa. This mixture was incubated for 20 minutes at 30° C. The reaction mixtures were filtered on Millipore filters, washed, dried, and counted. o-o control ribosomes, o---o control ribosomes minus ApUpG, x-x colicin E 3 treated ribosomes, x---x colicin E 3 treated ribosomes minus ApUpG.

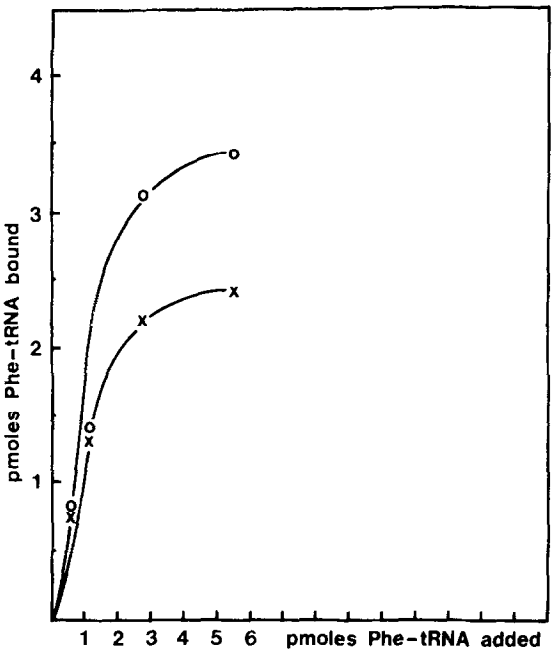

Fig. 4: EF-1 dependent binding of Phe-tRNA to 80 S ribosomes. The reaction mixtures and incubation were the same as described in Fig. 3 except that salt washed ribosomes were used, ApUpG was replaced by 0.2 A_{260} units poly-U and 30 µg of EF-1 were added. o-o control ribosomes, x-x colicin E 3 treated ribosomes.

through a sucrose gradient were mixed with untreated 40 S particles, the resulting 80 S ribosomes display a drastically reduced poly-phe synthesizing ability relative to the controls (Table 2). The apparent sensitivity of the 60 S subunits towards colicin E 3 is a novel feature which cannot be fully explained at the moment. It may either reflect a damage of their ribosomal RNA or merely a tight binding of colicin E 3 molecules to the large subunits which upon reassociation with 40 S particles cleave the 18 S RNA.

The experiments in this paper show that 1) colicin E 3 is active in mammalian as well as in bacterial systems. 2) Colicin E 3 inactivates mammalian 60 S subunits while leaving bacterial 50 S subunits unaffected. 3) All the other characteristics of colicin E 3 induced inhibition seem identical in the two systems.

Table 2

Action of colicin E 3 on isolated ribosomal subunits

Exp.No.	40 S	60 S	Phe incorporated (pmoles)	% activity
1	control	control	3.00	100
	colicin E 3	control	2.89	96
2	control	control	0.80	100
	control	colicin E 3	0.22	27

Experiment 1: 0.1 A_{260} units of untreated and 0.12 A_{260} units of colicin E 3 treated 40 S subunits were reassociated with 0.26 A_{260} units of 60 S particles. 0.23 A_{260} units of poly-U, 30 µg EF-1, 10 µg EF-2, 5.5 pmoles Phe-tRNA were added. 100 µl reaction mixture contained: 25 mM Tris.HCl pH 7.5, 80 mM KCl, 50-70 mM NH_4Cl, 5 mM magnesium acetate, 2 mM dithioerythritol, and 0.5 mM GTP. Reaction mixtures were incubated for 60 min. at 37° C. 0.1 ml of a 0.4 M ammonia was then added and the mixtures incubated again for another 30 minutes at 37° C. The hot TCA precipitable material was filtered over Millipore filters, washed, dried and counted.
Experiment 2: 0.27 A_{260} units of untreated and 0.25 A_{260} units of colicin E 3 treated 60 S subunits were reassociated with 0.1 A_{260} units of 40 S subunits and poly-phe synthesis was measured as described above. 4.66 pmoles of Phe-tRNA were added.

Table 3

Function of subunits from colicin E 3 treated 80 S ribosomes

Exp.No.	40 S	60 S	Phe incorporated (pmoles)	% activity
1	control	control	1.10	100
	control	colicin E 3	0.23	21
	colicin E 3	control	0.48	43
2	control	control	0.25	100
	colicin E 3	colicin E 3	0.02	< 10

After incubation with colicin E 3 80 S ribosomes were dissociated into subunits by lowering the magnesium concentration to 2.5 mM and increasing the KCl concentration to 500 mM. The subunits were isolated after sucrose gradient centrifugation. 0.1 A_{260} units control 40 S, 0.22 A_{260} units control 60 S, 0.1 A_{260} units 40 S and 0.23 A_{260} units 60 S from colicin E 3 treated 80 S ribosomes were reassociated and poly-phe synthesis was measured as described in Table 2.

References:

1. Senior, B.W., and Holland, I.B., Proc. Natl. Acad. Sci. US. 68, 959 (1971).
2. Bowman, C.M., Dahlberg, J.E., Ikemura, T., Konisky, J., and Nomura, M., Proc. Natl. Acad. Sci. US. 68, 964 (1971).
3. Boon, T., Proc. Natl. Acad. Sci. US. 68, 2421 (1971).
4. Bowman, C.M., Sidikaro, J., and Nomura, M., Nature New Biology 234, 133 (1971).
5. Sidikaro, J., and Nomura, M., Febs letters 29, 15 (1973).
6. Glick, J.M., Kerr, S.J., Gold, A.M., and Saemin, D., Biochemistry 11, 1183 (1972).
7. Drews, J., Grasmuk, H., and Weil, R., Eur. J. Biochem. 26, 416 (1972).
8. Felicetti, L., and Lipmann, F., Archives Biochem. Biophys. 125, 548 (1968).
9. Gillam, I., Blew, D., Warrington, R.C., von Tigerstrom, M., and Tener, G.M., Biochemistry 7, 3459 (1968).
10. Eggen, K.L., and Shatkin, A.J., J. Virology 9, 636 (1972).
11. Mathews, M.B., and Korner, A., Eur. J. Biochem. 17, 328 (1970).
12. Blobel, G., and Sabatini, D., Proc. Natl. Acad. Sci. US. 68, 390 (1971).
13. Leder, P., and Bursztyn, H., Biochem. Biophys. Res. Comm. 25, 233 (1966).
14. Turnowsky, F., and Högenauer, G., in preparation.
15. Boon, T., Proc. Natl. Acad. Sci. US. 69, 549 (1972).