Short Communications

Inhibition by Ricin of Protein Synthesis *in vitro*: 60S Ribosomal Subunit as the Target of the Toxin

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Poly(U)-directed polyphenylalanine synthesis by rat liver ribosomes is strongly inhibited by ricin. Experiments involving hybridization between subunits derived from normal and ricin-treated ribosomes demonstrate that the 60S subunit is the site of action of the toxin. The toxin inactivates the 60S subunit independently of the presence of the 40S subunit.

Ricin a toxic protein from the seeds of *Ricinus communis*, has been reported to be a powerful inhibitor of protein synthesis in cell-free extracts of rabbit reticulocytes (Olsnes & Pihl, 1972*a,b*) and in a system reconstituted from rat liver ribosomes and isolated EF 1* and EF 2 (Montanaro *et al.*, 1973). The toxin induces an irreversible modification of rat liver ribosomes, which become unable to support phenylalanine polymerization and to complement the GTPase activity of EF 2; no inhibition of the EF 1-catalysed binding of phenylalanyl-tRNA to ribosomes nor of the peptidyl-puromycin reaction used as a test for peptide-bond formation was observed (Montanaro *et al.*, 1973).

The results reported in the present communication indicate that the site of action of ricin is the 60S ribosomal subunit.

Experimental

Materials. The materials were obtained from commercial sources as indicated by Montanaro *et al.* (1973). L-[¹⁴C]Phenylalanyl-tRNA (0.137 μ Ci of [¹⁴C]phenylalanine/mg of tRNA; 385 mCi/mol of L-phenylalanine) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Methods. Ricin was purified as described by Moulé (1951). A protein fraction containing EF 1 and EF 2 was prepared from rat liver 'pH5 supernatant' by precipitation between 25 and 65% saturation with (NH₄)₂SO₄ as described by Felicetti & Lipmann (1968). Protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Rat liver polyribosomes were prepared as described by Staehelin & Falvey (1971). Ricin-treated ribosomes were prepared as described by Montanaro *et al.* (1973) by incubating polyribosomes at a final concentration of 3 *E*₂₆₀ units/ml in an incu-

bation medium containing 150 mM-NH₄Cl, 7.5 mM-magnesium acetate, 20 mM-Tris-HCl buffer, pH 7.5, 1 mM-dithiothreitol and 2 μ g of ricin/ml. Control ribosomes were incubated under the same ionic conditions, but in the absence of ricin. After 10 min at 37°C, ribosomes were spun down at 4°C in a Spinco 30 angle rotor (Beckman model L2-65K ultracentrifuge) operated for 3 h and 30 min at 78000 *g*_{av.}

Preparation of subunits from ricin-treated ribosomes and from control ribosomes was performed as described by Blobel & Sabatini (1971). The ribosomal pellets were resuspended in ice-cold distilled water at a final concentration of 50–100 *E*₂₆₀ units/ml. To 0.6 ml of ribosomal suspension 0.75 ml of salt-buffer solution and 0.15 ml of 0.01 M-puromycin dihydrochloride adjusted to pH 7.0 with 1 M-KOH were added. The final concentrations of buffer and salts were 50 mM-triethanolamine-HCl, pH 7.5, 1.5 mM-MgCl₂ and 500 mM-KCl. After 15 min at 0°C followed by 10 min at 37°C, each sample was layered over 25 ml of a 5–20% (w/v) linear sucrose gradient containing 50 mM-triethanolamine-HCl buffer, pH 7.5, 5 mM-MgCl₂, 500 mM-KCl and 1 mM-dithiothreitol. The gradients were centrifuged for 4 h and 30 min at 20°C in a Spinco SW25.1 rotor at 63600 *g*_{av.}. At the end of centrifugation the bottom of the tubes was punctured with a needle through which a 2 M-sucrose solution was injected at the rate of 1 ml/min by means of a peristaltic pump (LKB 12000 VarioPerpex Pump). The upward-displaced gradients were collected through a conical cap adapted at the top of the centrifuge tube in 0.6 ml fractions by means of a fraction collector (LKB 7000 UltraRac) equipped with a photometer (LKB 8300 Uvicord II) for continuous recording of absorbance at 253 nm. The fractions corresponding to the peaks of the 40S and 60S subunits were pooled separately and the subunits were sedimented at 4°C in the Spinco Ti 50 rotor operated at 105000 *g*_{av.} for 6 h.

Alternatively, 40S and 60S subunits were prepared

* Abbreviations: EF 1, elongation factor 1; EF 2, elongation factor 2; GTPase, guanosine triphosphatase.

directly from polyribosomes and then incubated separately, at final concentrations of 1.5 and 3 E_{260} units/ml respectively, with ricin (2 $\mu\text{g}/\text{ml}$). The ionic conditions during incubation were those described for ricin-treated ribosomes. Control 40S and 60S subunits were incubated similarly, but in the absence of ricin. After 10 min at 37°C, the incubation mixtures were layered over discontinuous sucrose gradients consisting of 7 ml of 0.7M-sucrose and 5 ml of 0.15M-sucrose, both containing 50mM-triethanolamine-HCl buffer, pH7.5, 5mM-MgCl₂, 500mM-KCl and 1mM-dithiothreitol. The subunits were sedimented through the gradients at 4°C in the Spinco 40 angle rotor operated at 78000g_{av} for 16h.

Poly(U)-directed polyphenylalanine synthesis catalysed by isolated and reassociated subunits was assayed in a reaction medium containing, in a final volume of 0.25ml, 150mM-NH₄Cl, 15mM-MgCl₂, 20mM-Tris-HCl buffer, pH7.5, 1mM-dithiothreitol, 2mM-GTP, 73 μg of [¹⁴C]phenylalanyl-tRNA, 200 μg of poly(U) and 250 μg of the protein fraction containing EF 1 and EF 2. Subunits were present in the amounts indicated in the legends to the Tables. After 40 min at 30°C, 0.25ml of 10% (w/v) trichloroacetic acid was added to each sample and the samples were heated at 90°C for 10 min. The hot-acid-insoluble radioactivity was collected on glass-fibre filters (Whatman GF/C). The filters were washed with 5% trichloroacetic acid and assayed for radioactivity in a Nuclear-Chicago mark I scintillation counter (counting efficiency approx. 60% for ¹⁴C) after addition of 6ml of ethylene glycol monomethyl ether and 10ml of scintillation fluid [0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene]. Counts were corrected to 100% efficiency.

Results and discussion

When isolated control subunits were tested separately and together for poly(U)-directed polyphenylalanine synthesis, the 40S subunit alone had no activity and the 60S subunit alone showed an activity which amounted to less than 7% of that displayed by the recombined subunits (Tables 1 and 2). Subunits isolated from ricin-treated ribosomes were inactive, when tested either separately or reassociated (Table 1). When each subunit derived from ricin-treated ribosomes was allowed to hybridize with the complementary subunit isolated from control ribosomes, polyphenylalanine synthesis was completely inhibited in the presence of 60S subunits derived from ricin-treated ribosomes complemented with control 40S subunits; on the other hand, the reassociation of control 60S subunits with 40S subunits derived from ricin-treated ribosomes led to functionally active ribosomes with a polyphenylalanine-synthesizing activity even higher than that of the recombined control subunits (Table 1).

These results clearly indicate the 60S ribosomal subunit as the target of ricin. Since, however, peptide-bond formation is unaffected by the toxin (Montanaro *et al.* 1973), the lesion induced by ricin in the 60S subunit must occur at a site distinct from the peptidyl-transferase centre, which catalyses peptide-bond formation and which is an integral part of the large ribosomal subunit (Monro *et al.*, 1969; Vazquez *et al.*, 1969). The action of ricin on rat liver ribosomes resembles that of thiostrepton (Pestka, 1970) and siomycin (Modolell *et al.*, 1971) on bacterial ribosomes: both antibiotics interact with the large ribosomal subunit, inhibiting translocation and the ribosome-

Table 1. Poly(U)-directed polyphenylalanine synthesis catalysed by subunits isolated from control and ricin-treated ribosomes

After incubation in the presence and in the absence of ricin, ribosomes were dissociated into subunits and the isolated subunits were tested for polyphenylalanine synthesis as described under 'Experimental'. In Expt. 1, 0.38 E_{260} unit of 40S and 1.14 E_{260} units of 60S subunits from control ribosomes and 0.40 E_{260} unit of 40S and 1.14 E_{260} units of 60S subunits from ricin-treated ribosomes were used. In Expt. 2, 0.30 E_{260} unit of 40S and 0.90 E_{260} unit of 60S subunits from control ribosomes and 0.30 E_{260} unit of 40S and 0.90 E_{260} unit of 60S subunits from ricin-treated ribosomes were used.

Source of subunit		Radioactivity incorporated (d.p.m.)	
40S	60S	Expt. 1	Expt. 2
Control	—	28	24
—	Control	115	70
Control	Control	1975	1037
Ricin-treated	—	28	27
—	Ricin-treated	46	82
Ricin-treated	Ricin-treated	63	118
Control	Ricin-treated	60	89
Ricin-treated	Control	4062	3342

Table 2. *Poly(U)-directed polyphenylalanine synthesis catalysed by isolated control subunits and by isolated subunits treated with ricin*

Isolated subunits incubated in the presence and in the absence of ricin were sedimented through discontinuous sucrose gradients and tested for polyphenylalanine synthesis as described under 'Experimental'. The results are means of two experiments with 0.41 E_{260} unit of control 40S, 1.26 E_{260} units of control 60S, 0.38 E_{260} unit of ricin-treated 40S and 1.18 E_{260} units of ricin-treated 60S subunits.

Subunit		Radioactivity incorporated (d.p.m.)
40S	60S	
Control	—	26
—	Control	146
Control	Control	3199
Ricin-treated	—	27
—	Ricin-treated	37
Ricin-treated	Ricin-treated	228
Control	Ricin-treated	137
Ricin-treated	Control	2916

linked GTPase activity of the bacterial supernatant factor involved in translocation but not the ribosomal peptidyl-transfer reaction. Ricin appears to be the corresponding tool for the study of peptide-bond formation uncoupled from the 60S ribosomal functions related to translocation in eukaryotic ribosomes. The finding that the rat liver 40S subunit is unaffected by ricin is consistent with the observation (Montanaro *et al.*, 1973) that the EF 1-catalysed binding of phenylalanyl-tRNA to ribosomes is unaffected by the toxin; in fact EF 1, in the presence of aminoacyl-tRNA and poly(U), has been reported to bind to isolated 40S subunits (Rao & Moldave, 1969).

Table 2 shows the results of experiments in which isolated subunits prepared from polyribosomes were incubated separately with ricin and tested for polyphenylalanine synthesis after centrifugation through

sucrose gradients. The activity of the 60S subunit was again severely inhibited when mixed either with ricin-treated 40S subunits or with the complementary control subparticle. Free ricin from the preincubation mixtures did not co-sediment with subunits through the sucrose gradients, since the ricin-treated 40S subunit was active when complemented with control 60S subunits (Table 2). Moreover, the addition of 60S ricin-treated subunits to control ribosomes did not modify their phenylalanine-polymerizing activity.

The experiments reported in Table 2 indicate that the simple preincubation with ricin is sufficient to inactivate the 60S subunit. This result confirms that, whatever the mechanism of ricin action may be, it does not require added cofactors (Montanaro *et al.*, 1973), not even the presence of the small ribosomal subunit.

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