Inhibition by Ricin of Protein Synthesis *in vitro*

INHIBITION OF THE BINDING OF ELONGATION FACTOR 2 AND OF ADENOSINE DIPHOSPHATE-RIBOSYLATED ELONGATION FACTOR 2 TO RIBOSOMES

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The binding of EF 2 (elongation factor 2) and of ADP-ribosyl-EF 2 to rat liver ribosomes is inhibited by ricin. This result suggests that the native enzyme and its ADP-ribose derivative have the same or closely related binding sites on the ribosome. The inhibition by ricin of the binding of EF 2 to ribosomes is consistent with the previous observation that ricin affects EF 2-catalysed translocation during polypeptide chain elongation.

Ricin, a toxic protein from the seeds of *Ricinus communis*, is a powerful inhibitor of protein synthesis in eukaryotic systems (Lin *et al.*, 1971; Olsnes & Pihl, 1972; Montanaro *et al.*, 1973). *In vitro* the toxin induces an irreversible modification of ribosomes (Olsnes *et al.*, 1973; Montanaro *et al.*, 1973), and precisely of the 60S subunit (Sperti *et al.*, 1973), which makes them unable to support polypeptide chain elongation (Olsnes & Pihl, 1972). Since the enzymic binding of aminoacyl-tRNA to ribosomes and peptide-bond formation were unaffected by the toxin, it was suggested that the modification of ribosomes induced by ricin impaired their activity in the translocation reaction catalysed by EF 2* (Montanaro *et al.*, 1973).

A reaction closely related to EF 2-dependent translocation is the binding of the enzyme to ribosomes in the presence of a guanine nucleotide (Skogerson & Moldave, 1968a; Bermek & Matthaei, 1971). ADP-ribosyl-EF 2, obtained by reacting the enzyme with diphtheria toxin and NAD⁺ (Honjo *et al.*, 1968) also binds to ribosomes, although otherwise inactive in protein synthesis (Bermek, 1972). The results reported in the present paper indicate that ribosomes treated with ricin are unable to bind both the native enzyme and its ADP-ribose derivative.

Experimental

Materials

Ricin was purified as described by Moulé (1951). Its molecular weight was assumed to be 65000 (Lin *et al.*, 1970). Diphtheria toxin, purified by the method of Pope & Stevens (1958), was obtained from the Istituto Sieroterapico e Vaccinogeno Toscano 'Scavo' (Siena, Italy). [8-³H]Puromycin (2 Ci/mmol),

* Abbreviations: EF 1, elongation factor 1; EF 2, elongation factor 2; A site, aminoacyl-tRNA site; P site, peptidyl-tRNA site; GTPase, guanosine triphosphatase.

[adenosine-¹⁴C]NAD⁺ (260 mCi/mmol) and [¹⁴C]-GDP (492 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. L-[¹⁴C]Phenylalanyl-tRNA (0.137 μ Ci of [¹⁴C]phenylalanine/mg of tRNA: 385 mCi/mmol of L-phenylalanine) was from New England Nuclear Corp., Boston, Mass., U.S.A. GTP and bovine serum albumin were products of Sigma Chemical Co., St. Louis, Mo., U.S.A., and poly(U) (potassium salt) was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

EF 1 and EF 2 were obtained from rat liver 'pH 5 supernatant' and were resolved from each other by adsorption on hydroxyapatite and chromatography on DEAE-cellulose as previously described (Montanaro *et al.*, 1973). The EF 2 protein fraction from the DEAE-cellulose step was further purified by chromatography on phosphocellulose as described by Raeburn *et al.* (1971). If necessary the chromatographic fractions containing EF 2 were concentrated in an Amicon ultrafiltration cell by using PM 30 membranes. The EF 2 solution was dialysed against 50 mM-Tris-HCl buffer, pH 7.5, containing 1 mM-dithiothreitol, and stored at -20°C.

Rat liver polyribosomes were prepared as described by Staehelin & Falvey (1971). Translocated ribosomes, i.e. ribosomes carrying their nascent peptidyl-tRNA at the P site (Baliga & Munro, 1972), were prepared by incubating freshly prepared polyribosomes with EF 2 and excess of GTP as previously described (Montanaro *et al.*, 1973). Non-translocated ribosomes, carrying peptidyl-tRNA mainly at the A site (Baliga & Munro, 1972), were prepared by incubating polyribosomes under similar conditions, but in the absence of EF 2 and GTP. Ricin-treated ribosomes were obtained by adding ricin at a final concentration of 2 μ g/ml to the incubation mixtures of both non-translocated and translocated ribosomes (Montanaro *et al.*, 1973). At the end of a 10 min incubation period at 37°C, the four ribosomal pre-

parations were spun down and ribosomes were washed twice through discontinuous sucrose gradients to remove all adherent EF 2 and GTP. The gradients consisted of 3 ml of 2M-sucrose and 4 ml of 0.7M-sucrose, both containing 20mM-Tris-HCl buffer, pH 7.5, 3mM-magnesium acetate, 1mM-dithiothreitol and 0.6M-NH₄Cl; NH₄Cl was omitted from the 2M-sucrose layer of the second washing. Each gradient was charged with 1 ml of ribosomal suspension and centrifuged at 4°C in the Spinco 40 angle rotor operated at 105000g_{av.} for 20h. The ribosomal pellets were rinsed, resuspended in water and stored in small portions at -20°C. Their activity remained unchanged for more than 20 days. Ribosome concentration was calculated from the E_{260} by assuming $E_{260}^{1\%}$ to be 145 and the molecular weight of the ribosome to be 4.1×10^6 (Baliga & Munro, 1972). With the latter value 1 mg of ribosomes corresponds approximately to 250 pmol.

Assays

[¹⁴C]ADP-ribosylation of EF 2 and the binding of [¹⁴C]ADP-ribosyl-EF 2 to ribosomes were performed in two-step experiments as described by Bermek (1972). ADP-ribosylation was carried out in reaction mixtures containing, besides EF 2, 50mM-Tris-HCl buffer, pH 7.5, 5mM-MgCl₂, 80mM-NH₄Cl, 10mM-dithiothreitol, 5 μM-[¹⁴C]NAD⁺ and diphtheria toxin at a final concentration of 170 μg/ml. After 15 min at 20°C the amount of [¹⁴C]ADP-ribosyl-EF 2 formed was measured on 0.1 ml samples to which 0.1 ml of 10% (w/v) trichloroacetic acid was added; the acid-insoluble radioactivity was collected on glass-fibre filters (Whatman GF/C), washed with 5% (w/v) trichloroacetic acid and measured. The binding of ADP-ribosyl-EF 2 to ribosomes was carried out in a second incubation: 0.1 ml samples of the ADP-ribosylation mixture were added to 4-5 E_{260} units of ribosomes suspended in 0.05 ml of 50mM-Tris-HCl buffer, pH 7.5, containing 5mM-MgCl₂, 80mM-NH₄Cl, 10mM-dithiothreitol and 1mM-GTP. After 5 min at 37°C, 0.14 ml of each sample was layered over 1.8 ml of 0.25M-sucrose containing 50mM-Tris-HCl buffer, pH 7.5, 25mM-KCl, 5mM-MgCl₂ and 7mM-2-mercaptoethanol (buffer A), and centrifuged at 4°C in a Spinco Ti 50 rotor operated at 105000g_{av.} for 150 min. The ribosomal pellets were rinsed twice with 8 ml of ice-cold water and resuspended in 2 ml of water. A portion (0.2 ml) was removed from each sample for the determination of ribosome concentration by measuring E_{260} . To the remaining 1.8 ml an equal volume of 10% (w/v) trichloroacetic acid was added and the acid-insoluble radioactivity was collected and measured. Samples without ribosomes were run as blanks.

When the binding of native EF 2 to ribosomes was investigated, diphtheria toxin and [¹⁴C]NAD⁺ were omitted from the first incubation. At the end of the

second incubation, 0.07 ml samples were layered over 1 ml of buffer A and centrifuged for 150 min at 4°C in a Spinco Ti 50 rotor at 105000g_{av.}. The whole supernatant was carefully collected and 0.4 ml was tested for ADP-ribosylation as described above in the presence of 5 μM-[¹⁴C]NAD⁺ and 42 μg of diphtheria toxin.

The binding of [¹⁴C]GDP to ribosomes mediated by the native enzyme was assayed in reaction mixtures containing, in 0.1 ml, 3.5 E_{260} units of ribosomes, 3 pmol of EF 2, 50mM Tris HCl buffer, pH 7.5, 5mM-MgCl₂, 80mM-NH₄Cl, 10mM-dithiothreitol and 10 μM-[¹⁴C]GDP (diluted with unlabelled carrier to a specific radioactivity of 100 mCi/mmol). GDP, which is as effective as GTP in promoting the binding of EF 2 to ribosomes (Bermek & Matthaei, 1971), was chosen as labelled nucleotide rather than GTP because of the different EF 2-dependent GTPase activity of control and ricin-treated ribosomes (Montanaro *et al.*, 1973). After 5 min at 37°C, the reaction mixtures were diluted with 5 ml of ice-cold 20mM-Tris-HCl buffer, pH 7.5, containing 20mM-KCl and 10mM-MgCl₂ and filtered immediately on Millipore filters (HA; average pore size 0.45 μm) soaked in the same buffer for 2 h before use. The filters were washed three times with 5 ml of cold buffer, dried and assayed for radioactivity. The EF 2-mediated binding was calculated by subtracting the amount of GDP bound to ribosomes alone and to EF 2 alone from the total amount bound when the two components were incubated together. Blanks, obtained by filtering 1 nmol of [¹⁴C]GDP in 0.1 ml reaction mixtures without EF 2 and ribosomes, were subtracted from all values.

Poly(U)-directed polyphenylalanine synthesis was performed as previously described (Montanaro *et al.*, 1973). The formation of peptidyl-puromycin was measured in assay systems containing, in 0.25 ml, 60mM-Tris-HCl buffer, pH 7.5, 6mM-MgCl₂, 300mM-NH₄Cl, 5 μM-[³H]puromycin and 125 μg of ribosomes. After 15 min of incubation at 37°C the samples were analysed for hot-acid-insoluble radioactivity (Skoger-son & Moldave, 1968b).

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer (counting efficiency approx. 75% for ¹⁴C and 36% for ³H) after adding to the samples 6 ml of ethylene glycol monomethyl ether and 10 ml 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

Some characteristics of the four ribosomal preparations are reported in Table 1. As previously

Table 1. *Activity of the ribosome preparations in phenylalanine polymerization and in the peptidyl-[³H]puromycin reaction*

The assay for poly(U)-directed phenylalanine polymerization contained (in 0.25 ml): 150 mM-NH₄Cl, 9 mM-magnesium acetate, 20 mM-Tris-HCl buffer, pH 7.5, 1 mM-dithiothreitol, 2 mM-GTP, 73 µg of [¹⁴C]phenylalanyl-tRNA (22000 d.p.m.), 200 µg of poly(U), 30 pmol of ribosomes, 25 µg of EF 2 protein and 90 µg of EF 1 protein. The assay mixture for the peptidyl-[³H]pyromycin reaction was as described in the Experimental section.

Ribosome preparation	Poly(U)-directed polymerization of [¹⁴ C]phenylalanine (radioactivity incorporated: d.p.m.)	[³ H]Puromycin reacted (pmol/100 pmol of ribosomes)
Non-translocated ribosomes		
Control	6208	1.66
Ricin-treated	1274	2.15
Translocated ribosomes		
Control	8680	9.20
Ricin-treated	1924	8.05

Table 2. *Binding of [¹⁴C]ADP-ribosyl-EF 2 to ribosomes and effect of ricin*

Ribosomes were tested for [¹⁴C]ADP-ribosyl-EF 2 binding as described in the Experimental section: GTP was omitted where indicated. In the 0.15 ml samples, 75 pmol of non-translocated ribosomes (control or ricin-treated), 77 pmol of translocated ribosomes (control or ricin-treated) and 22 pmol of ADP-ribosyl-EF 2 were present.

Ribosome preparation	Addition to assay mixture	[¹⁴ C]ADP-ribosyl-EF 2 bound (pmol/100 pmol of ribosomes)	
		GTP absent from assay mixture	GTP present in assay mixture
Non-translocated ribosomes			
Control	None	4.18	6.00
	Ricin (10 pmol)	—	3.10
	Ricin (100 pmol)	—	2.56
Ricin-treated	None	1.75	2.25
Translocated ribosomes			
Control	None	7.35	9.43
	Ricin (10 pmol)	2.98	2.62
	Ricin (100 pmol)	2.78	2.56
Ricin-treated	None	3.51	3.72

reported (Montanaro *et al.*, 1973) ribosomes pretreated with ricin, both translocated and non-translocated, were over 70% inhibited in polyphenylalanine synthesis. The remaining data in Table 1 show that approx. 2% of the non-translocated ribosomal population carried peptidyl-tRNA in the puromycin-reactive P site. This value increased fivefold after preincubation with EF 2 and GTP (translocated ribosomes) and the increase was scarcely affected by the presence of ricin in the preincubation mixture.

Table 2 shows the binding of ADP-ribosyl-EF 2 to the same ribosomal preparations. Under the conditions of the centrifugation assay (see the Experimental section) it was observed that: (i) control ribosomes bound significant [¹⁴C]ADP-ribosyl-EF 2 also when incubated in the absence of GTP; (ii) translocated ribosomes, i.e. ribosomes with the A site free, bound higher amounts of enzyme. The former observation is in agreement with that made by Gill *et al.* (1969) and by Chuang & Weissbach

(1972), who reported a significant binding of native EF 2 to ribosomes in the absence of added GTP, whereas the higher binding activity of translocated ribosomes is consistent with the increasing evidence that the presence of aminoacyl-tRNA or peptidyl-tRNA at the ribosomal A site interferes with the binding of EF 2 (Baliga & Munro, 1972; Richter, 1973) and of ADP-ribosyl-EF 2 (Üçer & Bermek, 1974) to ribosomes. In the presence of GTP the binding of ADP-ribosyl-EF 2 to both control translocated and non-translocated ribosomes was stimulated.

The data in Table 2 show that when ricin was present during the incubation of ADP-ribosyl-EF 2 with control ribosomes, or when ribosomes pretreated with ricin were tested, the GTP-dependent binding of ADP-ribosyl-EF 2 was completely abolished and the binding in the absence of GTP was greatly decreased.

A similar inhibitory effect of ricin was observed when the binding of native EF 2 was investigated.

Table 3. *Effect of ricin on the binding of native EF 2 and on the binding of [¹⁴C]GDP mediated by the native enzyme*

The assays for the binding of EF 2 (see the Experimental section) contained 47 pmol of non-translocated ribosomes and 37 pmol of translocated ribosomes. EF 2 bound to ribosomes in the absence and in the presence of GTP (expressed as pmol/100 pmol of ribosomes) was calculated by subtracting the amount of enzyme left in the supernatant from the amount titrated in duplicate samples without ribosomes run in parallel in the absence and in the presence of GTP. The assays for the EF 2-mediated binding of [¹⁴C]GDP (see the Experimental section) contained: 3 pmol of EF 2, 60 pmol of non-translocated ribosomes and 58 pmol of translocated ribosomes.

Ribosome preparation	EF 2 titrated in the supernatant (pmol)				EF 2 bound (pmol/ 100pmol of ribosomes)		[¹⁴ C]GDP bound (pmol/ 100pmol of ribosomes)
	GTP absent from assay mixture		GTP present in assay mixture		GTP absent from assay mixture	GTP present in assay mixture	
	No ribosomes	Ribosomes	No ribosomes	Ribosomes			
Non-translocated ribosomes							
Control	7.73	7.49	7.68	7.45	0.51	0.49	0.61
Ricin-treated	7.79	7.81	7.72	7.75	0.00	0.00	0.23
Translocated ribosomes							
Control	7.72	7.42	7.69	6.74	0.81	2.57	1.95
Ricin-treated	7.77	7.78	7.75	7.62	0.00	0.35	0.00

The amount of EF 2 bound was calculated as described in Table 3. Under the conditions of the assay, the binding of EF 2 to non-translocated ribosomes was hardly detectable, but a consistent GTP-dependent binding of the enzyme to translocated ribosomes was observed, which was greatly decreased when ribosomes had been pretreated with ricin (Table 3) or when ricin was present in the assay system in which control ribosomes were tested (results not shown).

Evidence has been reported that when EF 2 and GTP are incubated with ribosomes, GTP hydrolysis occurs and a ribosome-EF 2-GDP complex is obtained (Bermek & Matthaei, 1971; Chuang & Weissbach, 1972); the same complex is formed when the binding of EF 2 is mediated directly by GDP. The data in Table 3 for the EF 2-dependent binding of [¹⁴C]GDP to ribosomes, obtained by the Millipore-filter technique (see the Experimental section), show that the inhibitory effect of ricin on the binding of the native enzyme occurs also under conditions in which the formation of the complex is independent of the hydrolysis of GTP.

Discussion

In our experiments the effect of ricin on the binding of EF 2 to ribosomes was studied with the native enzyme or with the [¹⁴C]ADP-ribosyl-EF 2 obtained by treating the enzyme with [¹⁴C]NAD⁺ in the presence of diphtheria toxin. It was believed for some time that ADP-ribosyl-EF 2 was unable to form a complex with ribosomes (Kloppstech *et al.*, 1969; Everse *et al.*, 1970; Montanaro *et al.*, 1971). Bermek (1972), however, using milder conditions of centrifugation isolated a complex between ribosomes and

ADP-ribosyl-EF 2 and suggested (Üçer & Bermek, 1974) that the modified EF 2 bind to a ribosomal site specific for the active enzyme.

The use of radioactively labelled ADP-ribosyl-EF 2 allows a direct measure of the amount of the enzyme bound to ribosomes. On the other hand the binding of the native enzyme can be studied only indirectly. In the present experiments it was assayed either by measuring the amount of radioactive guanine nucleotide that complexes with ribosomes together with EF 2 (Bermek & Matthaei, 1971; Chuang & Weissbach, 1972), or by titrating the enzyme left in the supernatant after removal of the complex (Modolell *et al.*, 1971).

The results obtained clearly indicate that ricin inhibits the GTP-dependent binding of either EF 2 or ADP-ribosyl-EF 2 to ribosomes, thus supporting the suggestion of common or closely related binding sites on the ribosome for the active enzyme and for its ADP-ribose derivative (Üçer & Bermek, 1974).

The fact that ricin interferes with the binding of EF 2 to ribosomes is consistent with the observation that the toxin inactivates ribosomes *in vitro* by affecting the 60S ribosomal subunit (Sperti *et al.*, 1973), to which EF 2 has been observed to bind (McKeehan, 1972; Üçer & Bermek, 1974). The damage induced by ricin appears, however, limited, since the peptidyl-transferase centre itself, localized on the same subunit, is not affected (Montanaro *et al.*, 1973). Evidence is accumulating in favour of an enzymic mechanism of action of ricin (Olsnes & Pihl, 1972; Olsnes *et al.*, 1973; Montanaro *et al.*, 1973) which might affect either RNA or one (or more) component(s) of the 60S ribosomal subunit. Olsnes *et al.* (1973) claim evidence for a mechanism similar to that whereby colicin E 3 inactivates bacterial ribo-

somes, i.e. the cleavage of a nucleotide fragment from RNA. Hopefully ricin may help to elucidate the structural and functional organization of the components of eukaryotic ribosomes, particularly of those involved in GTPase and translocase activity.

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References

- Baliga, B. S. & Munro, H. N. (1972) *Biochim. Biophys. Acta* **277**, 368–383
- Bermek, E. (1972) *FEBS Lett.* **23**, 95–99
- Bermek, E. & Matthaei, H. (1971) *Biochemistry* **10**, 4906–4912
- Chuang, D. M. & Weissbach, H. (1972) *Arch. Biochem. Biophys.* **152**, 114–124
- Everse, J., Gardner, D. A., Kaplan, N. O., Galasinsky, W. & Moldave, K. (1970) *J. Biol. Chem.* **245**, 899–901
- Gill, D. M., Pappenheimer, A. M. & Baseman, J. B. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 595–602
- Honjo, T., Nishizuka, Y., Hayaishi, O. & Kato, I. (1968) *J. Biol. Chem.* **243**, 3553–3555
- Kloppstech, K., Steinbeck, R. & Klink, F. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 1377–1384
- Lin, J. Y., Tserng, K. Y., Chen, C. C., Lin, L. T. & Tung, T. C. (1970) *Nature (London)* **227**, 292–293
- Lin, J. Y., Liu, K., Chen, C. C. & Tung, T. C. (1971) *Cancer Res.* **31**, 921–924
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McKeehan, W. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1117–1122
- Modolell, J., Vazquez, D. & Monro, R. E. (1971) *Nature (London) New Biol.* **230**, 109–112
- Montanaro, L., Sperti, S. & Mattioli, A. (1971) *Biochim. Biophys. Acta* **238**, 493–497
- Montanaro, L., Sperti, S. & Stirpe, F. (1973) *Biochem. J.* **136**, 677–684
- Moulé, Y. (1951) *Bull. Soc. Chim. Biol.* **33**, 1461–1466
- Olsnes, S. & Pihl, A. (1972) *FEBS Lett.* **20**, 327–329
- Olsnes, S., Heiberg, R. & Pihl, A. (1973) *Mol. Biol. Rep.* **1**, 15–20
- Pope, C. G. & Stevens, M. F. (1958) *Brit. J. Exp. Pathol.* **39**, 139–149
- Raeburn, S., Collins, J. F., Moon, H. M. & Maxwell, E. S. (1971) *J. Biol. Chem.* **246**, 1041–1048
- Richter, D. (1973) *J. Biol. Chem.* **248**, 2853–2857
- Skogerson, L. & Moldave, K. (1968a) *J. Biol. Chem.* **243**, 5354–5360
- Skogerson, L. & Moldave, K. (1968b) *Arch. Biochem. Biophys.* **125**, 497–505
- Sperti, S., Montanaro, L., Mattioli, A. & Stirpe, F. (1973) *Biochem. J.* **136**, 813–815
- Stachelin, T. & Falvey, A. K. (1971) *Methods Enzymol.* **20**, 433–446
- Üçer, U. & Bermek, E. (1974) *FEBS Lett.* **38**, 161–165