Relationship between Elongation Factor 1- and Elongation Factor 2-Dependent Guanosine Triphosphatase Activities of Ribosomes

INHIBITION OF BOTH ACTIVITIES BY RICIN

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The elongation factor 1- and elongation factor 2-dependent GTPase (guanosine triphosphatase) activities of ribosomes are inhibited by ricin, a toxic protein known to inactivate the 60S ribosomal subunit. It is suggested that also in eukaryotic ribosomes a 'GTPase site', located on the larger subunit, is common to the two elongation factors.

The process of polypeptide-chain elongation in eukaryotic systems requires two soluble factors, EF1* and EF2. In the presence of a guanine nucleotide, EF1 catalyses the binding of aminoacyl-tRNA to the A site of ribosomes; when promoted by GTP, but not by GDP or GMP- $P(\text{CH}_2)_P$ (Skogerson & Moldave, 1968a; Hardesty *et al.*, 1969), the binding is followed by peptide-bond formation at the A site between the newly bound aminoacyl-tRNA and the nascent peptide chain present on the P site. EF2, which catalyses the translocation of the newly formed peptidyl-tRNA from the A site back to the P site, also binds to ribosomes in the presence of a guanine nucleotide [GTP, GDP, GMP- $P(\text{CH}_2)_P$, dGTP] (Chuang & Weissbach, 1972), although its activity in the cyclic process of elongation depends completely on GTP (Skogerson & Moldave, 1968b). When the single steps of elongation are investigated, hydrolysis of GTP accompanies the functional binding both of aminoacyl-tRNA (EF1- and aminoacyl-tRNA-dependent GTPase activity) and of EF2 (EF2-dependent GTPase activity) to ribosomes (Felicetti & Lipmann, 1968).

Recent experiments (Richter, 1973; Nombela & Ochoa, 1973) indicate that EF1 and EF2 interact in a mutually exclusive fashion with 80S ribosomes, suggesting that both factors compete for a common binding region on the ribosome. It is still open to discussion whether the interaction of the two factors with ribosomes occurs at the same site or whether it

involves separate sites with an overlapping region for which the two factors compete. The study of the mode of action of several inhibitors of polypeptide-chain elongation may help to clarify this feature of the ribosomal design.

In prokaryotic systems, thiostrepton and siomycin, two antibiotics which interact with the 50S ribosomal subunit (Weisblum & Demohn, 1970; Modolell *et al.*, 1971a), were initially classified as inhibitors of translocation since, without affecting the peptidyltransferase centre, they inhibited the nucleotide-dependent binding of EFG to ribosomes and the EFG-dependent GTPase reaction (Pestka, 1970; Modolell *et al.*, 1971a). Subsequently the antibiotics were shown to inhibit also the EFT-catalysed binding of aminoacyl-tRNA to ribosomes and the GTPase activity normally associated with this reaction (Modolell *et al.*, 1971b). From these results it was suggested (Modolell *et al.*, 1971b; Cundliffe, 1971) that bacterial 50S ribosomes possess a single 'GTPase site', sensitive to siomycin and thiostrepton, which is utilized alternatively by EFT and EFG during peptide-chain elongation.

Previous results from our laboratory indicated that the action of ricin on rat liver ribosomes resembles that of thiostrepton and siomycin on bacterial ribosomes: the toxin acts on the 60S subunit (Sperti *et al.*, 1973), inhibiting the binding of EF2 to ribosomes (Montanaro *et al.*, 1975) and the EF2-dependent GTPase reaction (Montanaro *et al.*, 1973), but does not affect the activity of the peptidyltransferase centre as assayed by the puromycin reaction (Montanaro *et al.*, 1973).

With the aim of clarifying the relationship between the two GTPase activities in eukaryotic ribosomes, a further investigation on the effect of ricin has been undertaken. The results reported here indicate that although ricin does not inhibit the functional binding of aminoacyl-tRNA to ribosomes, it does inhibit the

* Abbreviations: designation of the elongation factors of eukaryotic origin as EF1 and EF2 and of the respective prokaryotic factors as EF T (EF Tu and EF Ts) and EFG conforms to currently accepted nomenclature (Caskey *et al.*, 1972); A site, aminoacyl-tRNA site; P site, peptidyl-tRNA site; GTPase, guanosine triphosphatase; GMP- $P(\text{CH}_2)_P$, guanosine (β,γ -methylene) triphosphate.

GTPase activity normally associated with this reaction.

Experimental

Materials

L-[^{14}C]Phenylalanyl-tRNA (0.122 μCi of [^{14}C]phenylalanine/mg of tRNA; 385 mCi/mmol of L-phenylalanine) was purchased as charged tRNA from New England Nuclear Corp., Boston, Mass., U.S.A. This commercial product also contained tRNA species charged with nineteen other non-radioactive amino acids. [γ - ^{32}P]GTP (642 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Norit A (activated charcoal), GTP and GDP were products of Sigma Chemical Co., St. Louis, Mo., U.S.A. GMP- $P(\text{CH}_2)_P$ was from Miles Laboratories Inc., Kankakee, Ill., U.S.A., and poly(U) (potassium salt) was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Ricin was purified as described by Moulé (1951). EF1 and EF2 were obtained from rat liver 'pH5 supernatant' and were resolved from each other by adsorption on hydroxyapatite and chromatography on DEAE-cellulose as previously described (Montanaro *et al.*, 1973). The protein content was determined 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). Control ribosomes and ricin-treated ribosomes were prepared as described by Sperti *et al.* (1973) by incubating polyribosomes (3 E_{260} units/ml) in the absence and in the presence of ricin (2 $\mu\text{g}/\text{ml}$). After 10 min at 37°C, the ribosomes were spun down at 4°C in a Spinco 30 angle rotor (Beckman model L2-65K ultracentrifuge) operated for 3 h 30 min at 78000 $g_{av.}$. Ribosomes free of endogenous peptidyl-tRNA (puromycin-treated ribosomes) were obtained as described by Montanaro *et al.* (1973) by incubating polyribosomes (30 E_{260} units/ml) in a complete protein-synthesizing system (Staehelin & Falvey, 1971) containing 50 μg of puromycin/ml. After 40 min at 37°C, KCl was added to a final concentration of 0.5 M, and ribosomes were spun down as described above. The pellets of all ribosomal preparations were resuspended in a small volume of 20 mM-Tris-HCl buffer, pH 7.5, containing 3 mM-magnesium acetate and 1 mM-dithiothreitol, and washed twice through discontinuous sucrose gradients. The gradients consisted of 3 ml of 2 M-sucrose and 4 ml of 0.7 M-sucrose, both containing 20 mM-Tris-HCl buffer, pH 7.5, 3 mM-magnesium acetate, 1 mM-dithiothreitol and 0.6 M-NH $_4$ Cl; NH $_4$ Cl was omitted from the 2 M-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 105000 $g_{av.}$ for 20 h.

Ribosome concentration was calculated from the E_{260} by assuming $E_{1\text{cm}}^{1\%}$ to be 145.

Assays

Poly(U)-directed polyphenylalanine synthesis and EF2-dependent GTPase activity of ribosomes were assayed as described by Montanaro *et al.* (1973). The EF1-dependent GTPase activity of ribosomes was assayed as described by Felicetti & Lipmann (1968) in incubation mixtures containing, in 0.125 ml, 60 mM-Tris-HCl buffer, pH 7.5, 80 mM-NH $_4$ Cl, 6 mM-MgCl $_2$, 2 mM-dithiothreitol, 30 μg of poly(U), 40 μg of [^{14}C]phenylalanyl-tRNA and 3 nmol of [γ - ^{32}P]GTP (diluted with unlabelled GTP to a specific radioactivity of 50 d.p.m./pmol); ribosomes and EF1 were present in the amounts indicated in the legends to Tables. After 10 min at 37°C, the reaction was terminated by the addition of 1 ml of ice-cold 2.6% (w/v) trichloroacetic acid containing 1 mM-KH $_2$ PO $_4$, 0.06 mM-potassium acetate and 30 mg of Norit A. The mixture was shaken for 30 s on a Vortex mixer and centrifuged for 5 min at 2500 g in a Martin Christ Universal centrifuge at 2°C. Supernatants were filtered through Whatman no. 1 paper and 0.1 ml samples of the filtrates were assayed for radioactivity. EF1-dependent GTPase activity was calculated by subtracting the activities of ribosomes alone and of EF1 alone from the total activity observed when the two components were incubated together.

The EF1-dependent binding of phenylalanyl-tRNA to ribosomes was assayed in reaction mixtures containing, in 0.5 ml, 60 mM-Tris-HCl buffer, pH 7.5, 80 mM-NH $_4$ Cl, 6 mM-MgCl $_2$, 2 mM-dithiothreitol, 100 μg of poly(U), 160 μg of [^{14}C]phenylalanyl-tRNA and 500 μg of ribosomes. Assays also contained 0.5 mM-puromycin, as suggested by Ibuki & Moldave (1968). Incubations were carried out in the presence and in the absence of 0.2 mM-guanine nucleotide and 80 μg of EF1 protein. After 15 min at 37°C, the reaction mixtures were diluted with 5 ml of ice-cold 60 mM-Tris-HCl buffer, pH 7.5, containing 80 mM-NH $_4$ Cl and 6 mM-MgCl $_2$, and filtered immediately on Millipore filters (HA; average pore size 0.45 μm). The filters were washed with 3 $\times$ 5 ml of cold buffer and dissolved in scintillation fluid for radioactivity measurements.

When the EF1-dependent incorporation of phenylalanyl-tRNA into ribosomal nascent peptidyl-tRNA was investigated, puromycin was omitted from the reaction mixtures. At the end of incubation, 0.5 ml of 10% (w/v) trichloroacetic acid was added 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), washed with 5% trichloroacetic acid and measured.

Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer after adding to the samples 6 ml of methoxyethanol and 10 ml of toluene

containing 2,5-diphenyloxazole (0.4%) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.05%). When a single radioisotope was measured, the efficiency of counting was 75% for ^{14}C and 66% for ^{32}P ; in dual-labelling counting the amount of spill-over of the ^{14}C into the ^{32}P channel was negligible, and the counting efficiency for ^{32}P was 44%.

Results

Preincubation of ribosomes with ricin (ricin-treated ribosomes) or the addition of ricin (20 $\mu\text{g}/\text{ml}$) to control or puromycin-treated ribosomes leads to an inhibition of over 80% in poly(U)-directed polypeptide synthesis catalysed by EF1 and EF2. In previous reports (Montanaro *et al.*, 1973, 1975) it was suggested that the inhibition resulted from damage to ribosomes which, under the action of ricin, became unable to interact with EF2. This conclusion was supported by the observation that ricin inhibits

the EF2-dependent GTPase activity of ribosomes measured uncoupled from translocation (Montanaro *et al.*, 1973) and by direct evidence that the binding of EF2 to ribosomes is inhibited by the toxin (Montanaro *et al.*, 1975).

Table 1 compares the effect of ricin on the EF2-dependent and on the EF1-dependent GTPase activities of puromycin-treated ribosomes. In the presence of ribosomes and EF2 (Expt. 1), ricin considerably decreased the increment in GTP hydrolysis observed when ribosomes and EF2 were incubated together (EF2-dependent GTPase activity). In the presence of EF1 and ribosomes, no increment in GTP hydrolysis was observed (Expt. 2, line 3) unless phenylalanyl-tRNA and poly(U) were also present in the assay mixture (Expt. 2, line 1). This marked dependence on the presence of phenylalanyl-tRNA indicates that the EF1 preparation was not contaminated by EF2. In the presence of phenylalanyl-tRNA, the EF1-dependent GTPase activity

Table 1. *Effect of ricin on the EF2-dependent and on the EF1- and phenylalanyl-tRNA-dependent GTPase activity of ribosomes*

In Expt. 1, puromycin-treated ribosomes (265 μg) were tested for EF2-dependent GTPase activity in the absence and in the presence of ricin; 30 μg of EF2 protein was present. Expt. 2 is a parallel experiment in which the same ribosomes were assayed for EF1-dependent GTPase activity as described in the Experimental section; 100 μg of EF1 protein was present. The data are based on four independent replications of the two experiments.

Expt.	Enzyme	Phenylalanyl-tRNA +poly(U)	Ricin (40 $\mu\text{g}/\text{ml}$)	[γ - ^{32}P]GTP hydrolysed (pmol)			Enzyme-dependent GTPase activity of ribosomes	
				Ribosomes	Enzyme	Ribosomes +enzyme	(pmol of [γ - ^{32}P]- GTP hydrolysed)	Activity (%)
1	EF2	—	—	269	127	1195	799	100
		—	+	238	128	643	277	35
2	EF1	+	—	359	496	1907	1052	100
		+	+	297	406	882	179	17
		—	—	312	420	661	0	0

Table 2. *EF1- and phenylalanyl-tRNA-dependent GTPase activity of ribosomes in the presence of added ricin or after pretreatment with ricin*

EF1-dependent GTPase activity was assayed in the presence of phenylalanyl-tRNA and poly(U) as described in the Experimental section. In Expt. 1, 276 μg of control ribosomes, 278 μg of ricin-treated ribosomes and 80 μg of EF1 protein were present; in Expt. 2, 265 μg of puromycin-treated ribosomes and 100 μg of EF1 protein were present. The data are based on four independent replications of the two experiments.

Expt.	Ribosomes	Ricin (40 $\mu\text{g}/\text{ml}$)	[γ - ^{32}P]GTP hydrolysed (pmol)			EF1-dependent GTPase activity of ribosomes	
			Ribosomes	EF1	Ribosomes +EF1	(pmol of [γ - ^{32}P]GTP hydrolysed)	Activity (%)
1	Control	—	161	286	844	397	100
		+	123	279	567	165	42
	Ricin-treated	—	316	286	698	95	30
2	Puromycin-treated	—	297	444	1299	558	100
		+	217	317	790	256	46

was inhibited by ricin (Expt. 2, line 2) and the inhibition was higher than that observed in the parallel experiment in which the EF2-dependent GTPase activity of ribosomes was tested (Expt. 1). The inhibition of EF2-dependent GTPase activity in different experiments ranged from 30% to 65%, whereas the EF1-dependent GTPase activity was from 50% to 83% inhibited. The two inhibitions were obtained both when ricin was present in the assay system and when ribosomes isolated from preincubation mixtures containing ricin were tested (Montanaro *et al.*, 1973, and Table 2).

Table 3 shows that ricin does not inhibit the EF1-catalysed binding of [¹⁴C]phenylalanyl-tRNA to ribosomes and that the binding occurs not only in the presence of GTP, but also of its non-hydrolysable analogue GMP-*P*(CH₂)*P* and of GDP. The last two guanine nucleotides are known to be unable to support the incorporation of bound aminoacyl-tRNA into nascent peptides (Skogerson & Moldave, 1968*a*; Hardesty *et al.*, 1969). If the different effect of GTP with respect to the other nucleotides depends on the GTPase event, the inhibition by ricin of the EF1-

dependent GTPase activity of ribosomes should induce a decreased incorporation of bound [¹⁴C]-phenylalanyl-tRNA into nascent peptides. Table 4 shows that this is not the case. Thus either the hydrolytic step which brings about the liberation of P_i occurs after peptide-bond formation, or the hydrolysis of GTP is far in excess over that required for the binding of phenylalanyl-tRNA (Felicetti & Lipmann, 1968).

Discussion

It has been suggested that the two eukaryotic elongation factors EF1 and EF2 bind either to a unique site on the ribosome or to closely overlapping sites, the presence of each factor excluding the interaction of the other (Richter, 1973; Nombela & Ochoa, 1973). If the site were unique, a toxic substance which damages it should interfere with the correct interaction of both elongation factors. On the other hand, if there were two sites with an overlapping region, a toxic substance acting on one of them should interfere only partially with the activity of the other. In inactivation of ribosomes by ricin, both EF1- and EF2-dependent GTPase activities are inhibited. This inhibition is concomitant with the blockage of EF2-dependent translocation (Montanaro *et al.*, 1973) and of the binding of EF2 to ribosomes (Montanaro *et al.*, 1975), whereas the EF1-dependent binding of aminoacyl-tRNA occurs correctly so that it can be followed by peptide-bond formation (Table 4). Taken together, these observations suggest that the binding sites on the ribosome for EF1 and EF2 are distinct, with an overlapping region, which might well be a GTP-binding site ('GTPase site') which is utilized alternatively by the two enzymes.

Rao & Moldave (1969) have provided evidence that EF1 binds together with aminoacyl-tRNA and poly(U) to isolated 40S rat liver subunits and that this binding is independent of the presence of GTP, whereas the binding of EF1 to 80S ribosomes is markedly dependent on added nucleotide. On the other hand EF2 has been observed to bind to isolated

Table 3. *Effect of ricin and of different guanine nucleotides on the EF1-catalysed binding of [¹⁴C]phenylalanyl-tRNA to ribosomes*

Ribosomes (500 µg) isolated from preincubation mixtures carried out in the absence (control) or in the presence (ricin-treated) of ricin (2 µg/ml) were tested for [¹⁴C]-phenylalanyl-tRNA binding as described in the Experimental section. The data are based on three independent experiments.

Nucleotide	Radioactivity bound to ribosomes (d.p.m.)	
	Control ribosomes	Ricin-treated ribosomes
None	18	52
GTP	2396	2747
GMP- <i>P</i> (CH ₂) <i>P</i>	1876	2052
GDP	1669	2000

Table 4. *Effect of ricin and of guanine nucleotides on the binding of [¹⁴C]phenylalanyl-tRNA to ribosomes and on the incorporation of [¹⁴C]phenylalanyl-tRNA into nascent peptides*

The binding of [¹⁴C]phenylalanyl-tRNA to ribosomes (500 µg) was assayed as described in the Experimental section, but in the absence of 0.5 mM-puromycin. The amount of [¹⁴C]phenylalanine incorporated into nascent peptides was measured as the radioactivity insoluble in hot trichloroacetic acid under the same experimental conditions (see the Experimental section). The data are based on three independent experiments.

Ribosomes	Nucleotide	[¹⁴ C]Phenylalanyl-tRNA bound (d.p.m.)	[¹⁴ C]Phenylalanyl-tRNA incorporated (d.p.m.)
Control	GTP	3623	2200
	GMP- <i>P</i> (CH ₂) <i>P</i>	2985	94
Ricin-treated	GTP	3820	2172
	GMP- <i>P</i> (CH ₂) <i>P</i>	2805	55

60S subunits (McKeehan, 1972; Üçer & Bermek, 1974). These observations and the evidence that ricin, which inactivates the 60S ribosomal subunit (Sperti *et al.*, 1973), inhibits the ribosomal GTPase activity complemented by both elongation factors, allow us to localize the GTPase site on the larger ribosomal subunit and the EF1- and EF2-binding sites on the 40S and 60S subunits respectively. Moreover the GTPase site must be located very close to the EF2-binding site on the 60S subunit, since ricin affects not only the EF2-dependent GTPase activity of ribosomes (Montanaro *et al.*, 1973) but also the binding of EF2 itself in the absence of added GTP (Montanaro *et al.*, 1975).

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