Effect of α -sarcin and ribosome-inactivating proteins on the interaction of elongation factors with ribosomes

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α -Sarcin from *Aspergillus giganteus* and the ribosome-inactivating proteins (RIPs) from higher plants inactivate the 60 S ribosomal subunit. The former is an RNAase, whereas RIPs are *N*-glycosidases. The site of cleavage of RNA and that of *N*-glycosidic depurination are at one nucleotide distance in 28 S rRNA [Endo & Tsurugi (1987) J. Biol. Chem. **262**, 8128–8130]. The effect of α -sarcin and that of RIPs on the interaction of elongation factors with *Artemia salina* (brine shrimp) ribosomes have been investigated. α -Sarcin inhibits both the EF1 (elongation factor 1)-dependent binding of aminoacyl-tRNA and the GTP-dependent binding of EF2 (elongation factor 2) to ribosomes, whereas two of the RIPs tested, ricin from *Ricinus communis* (castor bean) and volkensin from *Adenia volkensii* (kilyambiti), inhibit only the latter reaction. EF2 protects ribosomes from inactivation by both α -sarcin and ricin. The EF1-binding site is affected only by α -sarcin. The sensitivity of this site to α -sarcin is increased by pretreatment of ribosomes with ricin. *A. salina* ribosomes were highly resistant to the third RIP tested, namely gelonin from *Gelonium multiflorum*. All four proteins tested have, however, a comparable activity on the rabbit reticulocyte-lysate system.

INTRODUCTION

α -Sarcin is a small protein extracted from the mould *Aspergillus giganteus* which inhibits protein synthesis and has RNAase activity (Schindler & Davies, 1977; Vázquez, 1979). Whereas it cleaves naked RNA at multiple sites (Endo *et al.*, 1983), in intact ribosomes it splits a specific fragment (α -fragment) from the large RNA of the large subunit by hydrolysing a single phosphodiester bond located, in rat ribosomes, between G⁴³²⁵ and A⁴³²⁶ of 28 S RNA (Endo & Wool, 1982). The site of action of α -sarcin is in a highly conserved sequence of the large ribosomal RNA (Wool, 1984) present also in *Escherichia coli* ribosomes, which, however, are inactivated by a concentration of the toxin 10-fold higher than that effective on eukaryotic ribosomes (Schindler & Davies, 1977). Treatment of eukaryotic ribosomes with α -sarcin inactivates the 60 S subunit. The impairment of protein synthesis is attributed to the inhibition of the elongation-factor-1 (EF1)-dependent binding of aminoacyl-tRNA to ribosomes (Fernández-Puentes & Vázquez, 1977; Endo & Wool, 1982), although inhibition of the binding of the binary complex elongation factor 2 (EF2)-GTP has also been reported (Conde *et al.*, 1978).

Ricin, which belongs to the group of 'ribosome-inactivating proteins' (RIPs) of plant origin (Roberts & Selitrennikoff, 1986; Stirpe & Barbieri, 1986), acts on the 60 S subunit (Sperti *et al.*, 1973) by a different mechanism, consisting of the cleavage of the *N*-glycosidic bond of A⁴³²⁴ in 28 S RNA of rat liver ribosomes (Endo & Tsurugi, 1987; Endo *et al.*, 1987). This enzymic activity is shared by other proteins of plant and bacterial origin,

which, unlike α -sarcin, specifically inactivate eukaryotic ribosomes (Endo & Tsurugi, 1987; Endo *et al.*, 1988a,b; Stirpe *et al.*, 1988). As in the case of α -sarcin, the step of elongation primarily inhibited by ricin has been controversial. Evidence from our laboratory indicates that ricin inhibits the binding of EF2 (Montanaro *et al.*, 1975; Sperti *et al.*, 1976) without affecting the EF1-dependent binding of aminoacyl-tRNA (Montanaro *et al.*, 1973; Sperti & Montanaro, 1979).

Thus two modifications occurring only at one nucleotide distance in 28 S rRNA appear to be followed by different functional effects: the impairment of EF2 binding by ricin and, primarily, that of the EF1-catalysed binding of aminoacyl-tRNA by α -sarcin. However, since these results have been obtained by independent groups, often using different protein-synthesizing systems, the difference in the functional correlates of the biochemical targets of these toxins may not be so substantial.

Previous observations from our laboratory have shown that the cleavage by α -sarcin of an α -fragment from *Artemia salina* (brine shrimp) ribosomes (which unlike rat liver ribosomes are naturally devoid of nascent peptides) does not require a pretreatment with puromycin or the presence of EDTA, and that ricin, added in stoichiometric amounts to the ribosomes, does not prevent the cleavage of the α -fragment by α -sarcin (Sperti *et al.*, 1986a). The site of the RNAase activity of α -sarcin is therefore not protected by the binding of ricin to ribosomes, nor are the substrate properties of the α -sarcin site lost after the enzymic modification induced by ricin.

In the present study the effects of α -sarcin and ricin on the EF1-dependent binding of aminoacyl-tRNA and on

Abbreviations used: RIP, ribosome-inactivating protein; EF1, elongation factor 1; EF2, elongation factor 2; ID₅₀, concentration causing 50% inhibition of protein synthesis.

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Table 1. Effect of inhibitors on the EF1-dependent binding of phenylalanyl-tRNA and on the binding of ADP-ribosyl-EF2 to *A. salina* ribosomes

Experiments were carried out in two steps. Expt. 1, EF1-dependent aminoacyl-tRNA binding, step 1: ribosomes (15 pmol) were incubated in the absence and in the presence of inhibitors for 5 min at 24 °C in 15 µl of 80 mM-Tris/HCl, pH 7.4, 80 mM-KCl, 5 mM-magnesium acetate and 2 mM-dithiothreitol containing 2 mM-NAD⁺ and 3 µg of diphtheria toxin fragment A; step 2: EF1 (45 µg of protein), 200 µg of poly(U), 22 pmol of [¹⁴C]phenylalanyl-tRNA and 0.5 µmol of GTP were added in 85 µl of 80 mM-Tris, pH 7.4, 100 mM-KCl, 9 mM-magnesium acetate and 2 mM-dithiothreitol; incubation was at 24 °C for 30 min; protein synthesis: NAD⁺ and diphtheria toxin fragment A were omitted from step 1 and 0.4 pmol of EF2 were added along with EF1 in step 2. Expt. 2, ADP-ribosyl-EF2 binding, step 1: ribosomes (534 pmol) were incubated in the absence and in the presence of inhibitors at 24 °C for 15 min in 600 µl of 80 mM-Tris/HCl (pH 7.4)/120 mM-KCl/7 mM-magnesium acetate/2 mM-dithiothreitol. Samples were layered over 1 ml of 0.15 M-sucrose in the same buffer and centrifuged for 2 h at 175000 g; pellets were resuspended in 100 µl of buffer and ribosomal concentration was measured from A_{260} , assuming $A_{260}^{1\%} = 125$ and 1 mg = 250 pmol; step 2: 27 pmol of ribosomes from step 1 were incubated in 150 µl of the same buffer containing 1 mM-GTP and 78 pmol of [¹⁴C]ADP-ribosyl-EF2; after 20 min at 24 °C samples were layered on 1.8 ml of 0.25 M-sucrose in the same buffer and centrifuged as above; pellets were resuspended in water, ribosome concentration estimated from the A_{260} and the [¹⁴C]ADP-ribosyl-EF2 bound measured from the acid-insoluble radioactivity collected on glass-fibre filters; protein synthesis: 2.5 pmol of ribosomes from step 1 were used for protein synthesis as described in Table 3; this lower amount of ribosomes relative to that used in the protein synthesis assays of Expt. 1 explains the higher elongation.

Preincubation additions	Expt. 1			Expt. 2		
	Phe-tRNA bound		Protein synthesis (pmol of phenylalanine incorporated/100 pmol of ribosomes)	ADP-ribosyl-EF2 bound		Protein synthesis (pmol of phenylalanine incorporated/100 pmol of ribosomes)
	(pmol/100 pmol of ribosomes)	Activity (%)		(pmol/100 pmol of ribosomes)	Activity (%)	
None	22.8	100		33.2	100	456.6
α-Sarcin	6.9	30		20.9	63	127.8
Ricin A chain	22.1	97		10.3	31	36.5
Volkensin A chain	19.9	87		18.3	55	68.5
Gelonin	21.7	95		34.5	104	461.2
						100
						28
						8
						15
						101

the binding of EF2 to *A. salina* ribosomes have been compared directly. A possible synergism in the action of the two proteins has been also investigated. The study has been extended to two other ribosome-inactivating proteins of plant origin, namely volkensin, a two-chain toxic lectin extracted from the roots of *Adenia volkensii* (kilyambiti) (Stirpe *et al.*, 1985), and gelonin, a single-chain RIP from *Gelonium multiflorum* (Stirpe *et al.*, 1980).

MATERIALS AND METHODS

Ricin, gelonin (M_r 30500) and volkensin were generously given by Professor F. Stirpe of this Department, and α -sarcin (M_r 17000) by Dr. D. M. Schuurmans (State of Michigan Department of Public Health, Lansing, MI, U.S.A.). The A chains of ricin (M_r 30000) and volkensin (M_r 29000) were prepared by dye-affinity chromatography by previously described methods (Sperti *et al.*, 1986b; Montanaro *et al.*, 1988). Diphtheria toxin, purified by the method of Pope & Stevens (1958), was generously supplied by the Istituto Sieroterapico e Vaccinogeno Toscano 'Sclavo', Siena, Italy; diphtheria toxin fragment A (M_r 24000) was prepared as described by Chung & Collier (1977).

[adenosine- ^{14}C]NAD $^+$ (260 mCi/mmol), L-[^{14}C]phenylalanine (513 mCi/mmol) and L-[^{14}C]leucine (348 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. tRNA $^{\text{Phe}}$ (Sigma Chemical Co., St. Louis, MO, U.S.A.) was charged with [^{14}C]phenylalanine by the method of Hultin & Näslund (1978).

Ribosomes and the 'S-105 supernatant' from *Artemia salina* undeveloped cysts were prepared as described by Sierra *et al.* (1974). Rabbit reticulocyte lysate was prepared as described by Allen & Schweet (1962). EF1 and EF2 were obtained from rat liver 'pH 5 supernatant' and resolved from each other as described by Montanaro *et al.* (1973). The molar concentration of EF2 was determined by [^{14}C]ADP-ribosylation in 100 μl mixtures containing 0.4 μM [^{14}C]NAD $^+$ as described by Rambelli *et al.* (1987). The [^{14}C]ADP-ribosyl-EF2 used in the binding assays was prepared by incubating approx. 2 μM -EF2 (as determined by the previous ADP-ribosylation assay) with 4 μM [^{14}C]NAD $^+$ in the presence of 1.8 μM diphtheria toxin fragment A. After 15 min at 20 $^{\circ}\text{C}$, non-radioactive NAD $^+$ was added to the final concentration of 0.15 mM and incubation was allowed to proceed for a further 5 min. This second incubation did not modify the amount of radioactivity incorporated into EF2 and ensured the complete conversion of EF2 to ADP-ribosyl-EF2. Protein synthesis with a rabbit reticulocyte lysate was allowed to proceed as described by Sargiacomo *et al.* (1983), and poly(U)-directed polymerization of phenylalanine with ribosomes from *Artemia salina* undeveloped cysts was measured as described by Barbieri *et al.* (1980). EF1-dependent phenylalanyl-tRNA binding was carried out essentially as described by Sperti *et al.* (1976), with NAD $^+$ and diphtheria toxin fragment A present in the preincubation mixture of ribosomes in order to inactivate possible traces of contaminating EF2. ADP-ribosyl-EF2 binding to ribosomes was essentially as described by Montanaro *et al.* (1975). Hot-acid-insoluble radioactivity was collected on glass-microfibre filters (Whatman GF/C) as described by Montanaro *et al.* (1978) and, after addition of 5 ml of Ready-Gel (Beckman Instruments), measured in a LKB liquid-

scintillation counter. All experimental details are in the legends to the Tables and the Figure.

RESULTS

Table 1 compares the effect of α -sarcin and RIPs on the EF1-dependent binding of aminoacyl-tRNA and on

Table 2. Effect of ricin on the inhibition of EF1-dependent binding of aminoacyl-tRNA by α -sarcin

The aminoacyl-tRNA binding assay was performed as described in Table 1. Step 1 was carried out in the absence and in the presence of ricin A chain (0.25 pmol). In step 2, α -sarcin (15 pmol) was present where indicated.

Additions		Phe-tRNA bound	
Step 1	Step 2	(pmol/100 pmol of ribosomes)	Activity (%)
None	None	23.2	100
Ricin A chain	None	23.1	100
None	α -Sarcin	12.8	55
Ricin A chain	α -Sarcin	3.9	17

Fig. 1. Ability of EF2 to protect ribosomes from inactivation by ricin and α -sarcin

A. salina ribosomes (10 pmol) were preincubated 15 min at 24 $^{\circ}\text{C}$ in 45 μl of 80 mM-Tris/HCl (pH 7.4)/120 mM-KCl/7 mM-magnesium acetate/2 mM-dithiothreitol (buffer 1), containing 1 mM-GTP and, where indicated, 31.5 pmol of EF2. Inhibitors were then added in 5 μl of buffer 1 at the indicated final concentrations (nm) and ribosomes were incubated for a further 5 min at 24 $^{\circ}\text{C}$. Finally, 10 μl were withdrawn and protein synthesis was assayed as described in the legend to Table 3. Values are given as percentages of control activity; the amount of radioactivity incorporated in controls was 7120 d.p.m. in the absence and 12270 d.p.m. in the presence of EF2. ■, +EF2; □, -EF2.

the GTP-dependent binding of ADP-ribosyl-EF2 to *A. salina* ribosomes. In Expt. 1, under conditions in which α -sarcin and RIPs (except gelonin; see below) produced an approx. 80% inhibition of polyphenylalanine synthesis, the EF1-dependent binding of phenylalanyl-tRNA was inhibited only by α -sarcin. In contrast, in Expt. 2, the binding of ADP-ribosyl-EF2 was markedly affected by all inhibitors except gelonin (see below).

As shown in Table 2, ricin, although unable by itself to affect the aminoacyl-tRNA binding reaction, considerably enhanced the inhibitory effect of α -sarcin on this reaction. Thus the modification of 28 S rRNA induced by ricin not only does not inhibit the RNAase activity of α -sarcin (Sperti *et al.*, 1986a; Stirpe *et al.*, 1988), but makes 28 S rRNA a better substrate for this toxin.

Fig. 1 shows that EF2 protects *A. salina* ribosomes from the inactivation by ricin A chain and, although to a lesser extent, also from the inactivation by α -sarcin.

The observation that gelonin had no effect on the elongation cycle of *A. salina* ribosomes (Table 1) prompted a comparison of the ID₅₀ (concentration causing 50% inhibition) of the four proteins in the cell-free system from *A. salina* and in the rabbit reticulocyte-lysate system, which is the standard test for the screening of plants for the presence of RIPs (Barbieri & Stirpe, 1982). The results are reported in Table 3. All four proteins were very active as inhibitors of the rabbit

reticulocyte system, the ID₅₀ ranging from a minimum of 0.09 nM for α -sarcin to a maximum of 0.3 nM for volkensin. Large differences in sensitivity were, however, observed with the *A. salina* system: ricin had almost the same ID₅₀ as in the reticulocyte system; volkensin and α -sarcin were, respectively, 10 and 30 times less active; gelonin was completely ineffective, even when tested at a concentration 600 times higher than the ID₅₀ in the rabbit reticulocyte system. This insensitivity was observed in the presence of both *A. salina* (Table 1, Expt. 2, and Table 3) and rat liver (Table 1, Expt. 1) EFs.

DISCUSSION

The protective effect of EF2 against the action of ricin A chain has been taken as evidence that the target site of ricin on ribosomes is overlapping, or is very close to, the binding site of EF2 (Fernández-Puentes *et al.*, 1976). However, ricin, although sparing the binding site for EF1, assayed both as EF1-dependent binding of aminoacyl-tRNA (Montanaro *et al.*, 1973; Sperti & Montanaro, 1979) and as direct binding of radioiodinated EF1 (Nilsson & Nygård, 1986), inactivates the ribosomal GTPase activity complemented by both EFs (Benson *et al.*, 1975; Sperti *et al.*, 1975). It was therefore suggested (Sperti *et al.*, 1975) that the binding sites on the ribosome for EF1 and EF2 are distinct, with an overlapping region, the 'GTPase site', utilized alternately by the two enzymes. This site, together with the binding site for EF2, would be the target region of ricin.

On the other hand, inhibition both of the EF1-dependent binding of aminoacyl-tRNA and of the EF1-dependent GTPase activity (Fernández-Puentes & Vázquez, 1977; Hobden & Cundliffe, 1978) is consistently reported in the case of α -sarcin. Moreover, there is circumstantial evidence that the presence of aminoacyl-tRNA or peptidyl-tRNA on ribosomes protects 28 S rRNA from cleavage by α -sarcin (Endo & Wool, 1982). All these results led to the conclusion that the target site of α -sarcin on ribosomes is involved in the EF1-dependent binding of aminoacyl-tRNA (Endo *et al.*, 1983). The present observations, that α -sarcin diminishes the affinity of ribosomes for EF2 and that EF2 protects ribosomes against inactivation by α -sarcin, indicate that the damage done by α -sarcin spreads from the EF1-binding site to that of EF2, probably including the common 'GTPase site'. Caution must, however, be used in correlating the specific molecular modifications produced by these toxins in RNA with the specific functional damages appearing in ribosomes, since limited enzymic attack of rRNA can influence ribosomal structure at a distance, modifying the interactions between rRNAs or between RNA domains and ribosomal proteins. The inactivation of ribosomes by ricin is in fact associated with a destabilization of the 60 S ribosomal subunit demonstrable as an increased thermal release of the L5·5 S rRNA complex (Paleologue *et al.*, 1986), and the effect of α -sarcin is associated with a weakening of the interaction between 28 S and 5.8 S rRNA (Walker *et al.*, 1983).

A. salina ribosomes, which are sensitive to the RIPs from *Phytolacca americana* (pokeweed) (Irvin, 1975), *Ricinus communis* (Sperti *et al.*, 1976), *Adenia digitata* (Montanaro *et al.*, 1978), *Momordica charantia* (bitter pear melon) (Barbieri *et al.*, 1980) and *Adenia volkensii* (the present data), show a remarkable resistance to

Table 3. ID₅₀ of the inhibitors

ID₅₀ (concentration of inhibitor causing 50% inhibition of protein synthesis) values were calculated by the least-squares method applied to the linear regression between fractional activity and log(inhibitor concentration). Protein synthesis with the rabbit reticulocyte lysate was measured in reaction mixtures containing, in a final volume of 62.5 μ l, 10 mM-Tris/HCl, pH 7.4, 100 mM-ammonium acetate, 2 mM-magnesium acetate, 1 mM-ATP, 0.2 mM-GTP, 15 mM-creatine phosphate, 3 μ g of creatine kinase, 0.05 mM amino acids (minus leucine), 0.1 μ Ci of L-[¹⁴C]leucine, the appropriate amount of inhibitor and 25 μ l of reticulocyte lysate (corresponding to 2.5 pmol of ribosomes). After 7 min at 28 °C, 1 ml of 0.1 M-KOH was added and the samples were decolorized with two drops of 35% (w/v) H₂O₂. The acid-insoluble radioactivity was collected on glass-fibre filters and measured. Poly(U)-directed phenylalanine polymerization with *A. salina* ribosomes was carried out at 24 °C in 100 μ l of 80 mM-Tris/HCl (pH 7.4)/120 mM-KCl/7 mM-magnesium acetate/2 mM-dithiothreitol/2 mM-GTP, containing 22 pmol of [¹⁴C]phenylalanyl-tRNA, 80 μ g of poly(U), 40 μ g of 'S-105 supernatant' and 2.5 pmol of ribosomes. After 20 min the reaction was stopped and the hot-acid-insoluble radioactivity was measured.

Inhibitor	ID ₅₀ (nM)	
	Rabbit reticulocyte lysate	<i>Artemia salina</i> system
α -Sarcin	0.09	2.83
Ricin A chain	0.15	0.20
Volkensin A chain	0.30	3.13
Gelonin	0.26	No inhibition up to 150

gelonin. A great variability in the sensitivity to RIPs has already been observed among ribosomes from different plant species (Battelli *et al.*, 1984) and from protozoa (Cenini *et al.*, 1987, 1988). The present data indicate that a marked variability exists also among metazoa.

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