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Ricin and Modeccin do not Inhibit the Elongation Factor 1-Dependent Binding of Aminoacyl-tRNA to Ribosomes

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Ricin and modeccin do not affect the total number of ribosomes to which phenylalanyl-tRNA becomes bound in the EF 1-dependent reaction. Previous inconsistencies resulted from the use of the nitrocellulose-filter technique, which overestimates the number of control ribosomes engaged in the binding reaction if trace amounts of EF 2 contaminate the ribosomal preparations.

Ricin, a lectin present in the seeds of *Ricinus communis*, and modeccin, a protein from the roots of *Adenia digitata*, are two very potent toxic proteins. Both toxins inhibit protein synthesis *in vitro* at the level of polypeptide-chain elongation (Olsnes & Pihl, 1972; Montanaro *et al.*, 1973; Montanaro *et al.*, 1978) by inactivating the 60S ribosomal subunit (Sperti *et al.*, 1973; Benson *et al.*, 1975; Montanaro *et al.*, 1978) in such a way that EF 2 will no longer bind efficiently to ribosomes (Carrasco *et al.*, 1975; Montanaro *et al.*, 1975; Nolan *et al.*, 1976; Montanaro *et al.*, 1978). Although there is full agreement that ricin inhibits the binding of EF 2 to ribosomes and affects both the EF 2- and EF 1-dependent GTPase activities of ribosomes (Benson *et al.*, 1975; Sperti *et al.*, 1975), its effect on the EF 1-dependent binding of phenylalanyl-tRNA is still a source of controversy. Carrasco *et al.* (1975) and Fernandez-Puentes *et al.* (1976) described experiments in which ricin strongly inhibited the EF 1- and GTP-dependent binding of [¹⁴C]phenylalanyl-tRNA to rabbit reticulocyte ribosomes, whereas Nolan *et al.* (1976), working with ribosomes from Krebs II mouse ascites cells, found the binding to be unaffected. In all cases the binding was measured by the nitrocellulose-filter technique. Using the same technique in our laboratory, Montanaro *et al.* (1973) and Sperti *et al.* (1975) found that ricin had little influence on the EF 1- and GTP-dependent binding of [¹⁴C]phenylalanyl-tRNA to rat liver ribosomes measured in the presence of puromycin. A slight inhibition occurred when puromycin was omitted from the binding assay, but disappeared when GTP was replaced by its non-hydrolysable analogue p[CH₂]ppG (Sperti *et al.*,

1976). With *Artemia salina* ribosomes, the binding was unaffected when ribosomes were prepared as described by Zasloff & Ochoa (1971) and treated with NAD⁺ in the presence of diphtheria toxin to inactivate contaminating EF 2 (Sperti *et al.*, 1976). With *A. salina* ribosomes prepared by the method of Sierra *et al.* (1974), in which no EF 2 was detectable by [¹⁴C]ADP-ribosylation, the binding reaction, tested without previous ADP-ribosylation of the ribosomal preparations, was inhibited by up to 40% by both ricin and modeccin (Montanaro *et al.*, 1978). However, the presence of tRNA^{Phe}, which stimulates the binding of EF 1 to ribosomes (Grasmuk *et al.*, 1976), greatly diminished the inhibition. This effect, taken together with other considerations, led us to suggest (Montanaro *et al.*, 1978) that trace amounts of contaminating EF 2, although not detectable by [¹⁴C]-ADP-ribosylation, were still present in the ribosomal preparations and catalysed the formation of oligopeptides of phenylalanine not measurable as hot-acid-insoluble radioactivity, and therefore estimated by the nitrocellulose-filter technique as [¹⁴C]phenylalanyl-tRNA bound to ribosomes. It was pointed out that the presence of these oligopeptides would lead to an overestimate of the number of control ribosomes carrying phenylalanyl-tRNA, and that inhibition of their formation by inhibitors of translocation would simulate an inhibition of the EF 1-dependent binding reaction.

In the present paper the amount of oligopeptide formed during the binding assay carried out with *A. salina* ribosomes prepared as described by Sierra *et al.* (1974) has been measured by a chromatographic procedure. The results obtained indicate that neither ricin nor modeccin inhibits the EF 1-dependent binding of [¹⁴C]phenylalanyl-tRNA, but that they are specific inhibitors of translocation.

Abbreviations used: EF 1, elongation factor 1; EF 2, elongation factor 2; p[CH₂]ppG, guanosine [β -methylene]triphosphate; GTPase, guanosine triphosphatase.

Experimental

Materials and determinations

KCl-washed *A. salina* 80S ribosomes were prepared from undeveloped cysts as described by Sierra *et al.* (1974). EF 1 was obtained from rat liver 'pH5 supernatant' and resolved from EF 2 as described by Montanaro *et al.* (1973). Modeccin was prepared as described by Gasperi-Campani *et al.* (1978). Ricin was prepared as described by Nicolson & Blaustein (1972) and Nicolson *et al.* (1974). Cellex-BD (benzoylated DEAE-cellulose) was from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals were obtained from the sources given in Montanaro *et al.* (1978). The concentration of ribosomes was calculated from the A_{260} ($A_{1\text{cm}}^{1\%} = 125$; 1 mg of ribosomes = 250 pmol). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Binding assay

The EF 1-dependent binding of [^{14}C]phenylalanyl-tRNA to KCl-washed ribosomes was carried out as previously described (Montanaro *et al.*, 1978), both with uncharged ribosomes and with poly(U)-programmed ribosomes charged with tRNA^{Phe}. At the end of the binding assay (see the legend to Table 1), three 100 μl portions were withdrawn from the reaction mixtures. On one of them, total ribosome-bound [^{14}C]phenylalanine (Millipore-filter technique) and on the second polyphenylalanine synthesis (hot-trichloroacetic acid-insoluble radioactivity) were measured as previously described (Montanaro *et al.*, 1978). To the third 100 μl portion, KOH was added to a final concentration of 0.1M, and hydrolysis was allowed to proceed at room temperature for 30 min. After hydrolysis the samples were applied to small Cellex-BD columns (0.5 cm $\times$ 2 cm) and mono-, di- and tri-phenylalanine were separately eluted as described by Pestka (1971). Since resolution was essentially complete, it was not necessary to remove the unbound [^{14}C]phenylalanyl-tRNA before the chromatographic analysis. Bound monophenylalanyl-tRNA was calculated by subtracting from the radioactivity present in the monophenylalanine peak the radioactivity not retained on the Millipore filters during the assay of total bound radioactivity. The amounts of di- and tri-phenylalanine formed during the binding assay were obtained directly from the radioactivity present in the corresponding peaks.

Results and Discussion

The chromatographic analysis of the products of the binding reaction (Table 1) showed that in all cases the sum of the radioactivities present as mono-, di- and tri-phenylalanine accounted for over 90% of the total bound radioactivity. The residual radioactivity

Table 1. EF 1-dependent binding of [^{14}C]phenylalanyl-tRNA to KCl-washed *A. salina* ribosomes: effect of modeccin and ricin on the products of the binding reaction

KCl-washed ribosomes (228 pmol) were preincubated for 5 min at 24°C both in the absence and in the presence of inhibitors (20 μg). The experiments were then carried out in three steps. Step 1: 76 pmol of control or treated ribosomes were incubated for 10 min at 24°C in 200 μl of 80 mM-Tris/HCl buffer (pH 7.4) containing 80 mM-KCl, 10 mM-magnesium acetate, 2 mM-dithiothreitol and 800 μg of poly(U); 0.24 μg unit of tRNA^{Phe} was present where indicated. Step 2: 80 μl of EF 1 (200 μg of protein) was added and the samples were again incubated for 10 min at 24°C. Step 3: 2 μmol of GTP and 100 pmol of [^{14}C]phenylalanyl-tRNA were added in 120 μl of 130 mM-Tris/HCl buffer (pH 7.4)/200 mM-KCl/12 mM-magnesium acetate/3 mM-dithiothreitol; after 30 min of incubation at 24°C, 100 μl samples were withdrawn from the reaction mixtures. The determination of total ribosome-bound radioactivity, of hot-acid-insoluble radioactivity and the analysis of the products of the binding reaction were performed as described in the Experimental section.

[¹⁴ C]Phenylalanine bound (pmol)/100pmol of ribosomes																
Expt.	Inhibitor	tRNA ^{Phe}	Total (retained on Millipore filters)		Hot-acid- insoluble (pmol)	Products of the binding reaction										Actual number of ribosomes engaged in the binding reaction (pmol/100pmol)
			(pmol)	(% of control activity)		Monophenylalanine		Diphenylalanine		Triphenylalanine		Higher phenylalanine peptides				
						(pmol)	(% of total)	(pmol)	(% of total)	(pmol)	(% of total)	(pmol)	(% of total)			
1	None	—	22.62	100	1.60	3.03	13	14.98	66	3.23	14	1.33	6	11.6		
	Modeccin	—	15.13	67	0.71	9.69	67	2.93	20	0.89	6	0.94	7	11.5		
	Ricin	—	14.87	66	0.81	9.87	66	3.06	21	0.77	5	1.18	8	11.7		
2	None	+	50.26	100	1.93	16.74	33	27.36	54	5.14	10	1.10	2	32.1		
	Modeccin	+	46.81	93	0.90	42.93	89	3.77	8	0.94	2	0.73	1	45.1		
	Ricin	+	47.07	94	0.78	42.16	90	3.35	7	0.75	1	0.79	2	44.1		

could be eluted from the Cellex-BD columns with ethanolic 0.5M-KOH, which hydrolyses the benzoyl residues and liberates higher phenylalanine peptides (Pestka, 1971). The radioactivity eluted with this solvent (approx. 1pmol of phenylalanine/100pmol of ribosomes) was of the same order of magnitude as the amount of hot-acid-insoluble radioactivity (third column of Table 1). The formation of small amounts of hot-acid-insoluble radioactivity by control ribosomes during the binding assay led us previously to suspect (Montanaro *et al.*, 1978) the formation of even greater amounts of oligopeptides of phenylalanine, not measurable as hot-acid-insoluble radioactivity, but retained on Millipore filters. Table 1 shows that in the absence of inhibitors the presence of di- and tri-phenylalanine leads to an almost 2-fold overestimate of the number of ribosomes carrying monophenylalanyl-tRNA, as judged from the data obtained by the Millipore-filter technique. Thus in Expt. 1, the actual number of control ribosomes engaged in the binding reaction (neglecting the few ribosomes with long-chain peptides) is $[3.03 + (14.98/2) + (3.23/3)] = 11.6$, in contrast with the value of 22 given by the Millipore-filter technique. In Expt. 2, similar calculations lead to a value of 32, in contrast with a value of 50 given by the Millipore-filter technique.

In agreement with previous results (Montanaro *et al.*, 1978), the presence of modeccin or ricin inhibited to some extent the total radioactivity bound to ribosomes, the inhibition being greater in the absence than in the presence of tRNA^{Phe}. However, as shown in Table 1, a modification of the distribution of bound radioactivity occurred, the formation of di- and tri-

phenylalanine being drastically decreased and the amount of bound monophenylalanyl-tRNA increased. The consequence is that the number of ribosomes engaged in the binding reaction is unaffected by modeccin and ricin in the absence of tRNA^{Phe} (Expt. 1), or is even increased in the presence of tRNA^{Phe} (Expt. 2). This increase induced by modeccin and ricin (Table 1, last column of Expt. 2), as well as the decreased inhibitory effect of the toxins on total ribosome-bound radioactivity in the presence of tRNA^{Phe} (Montanaro *et al.*, 1978; and first column of Table 1), can be easily explained if we assume that, under the experimental conditions used, the presence of tRNA^{Phe} makes [¹⁴C]phenylalanyl-tRNA limiting in the reaction mixtures containing control ribosomes. Since modeccin and ricin prevent the synthesis of di- and tri-phenylalanine, more [¹⁴C]-phenylalanyl-tRNA is left available for its EF 1-dependent binding to tRNA^{Phe}-stimulated ribosomes. That this is the correct interpretation is supported by the data reported in Table 2 (Expt. 1). In the presence of tRNA^{Phe} an increase in the ratio of [¹⁴C]phenylalanyl-tRNA to ribosomes (obtained by either decreasing the number of ribosomes or increasing the amount of [¹⁴C]phenylalanyl-tRNA) induces an increase in the total radioactivity (pmol of phenylalanine/100pmol of ribosomes) bound to control ribosomes. The total radioactivity bound to ricin- or modeccin-treated ribosomes is much less affected, and the inhibition induced by the toxins reaches the same value of approx. 30% observed in the absence of tRNA^{Phe} (see Table 1).

The results reported in the present paper clearly indicate that neither modeccin nor ricin inhibits the

Table 2. EF 1-dependent binding of [¹⁴C]phenylalanine to KCl-washed *A. salina* ribosomes: effect of different concentrations of ribosomes, [¹⁴C]phenylalanyl-tRNA and poly(U) on the inhibition by modeccin and ricin

The experiments were carried out as described in the legend to Table 1, except that 100µl reaction mixtures were used, since only total ribosome-bound radioactivity was measured. Expt. 1 was carried out with 200µg of poly(U) and 0.06A₂₆₀ unit of tRNA^{Phe}; ribosomes and [¹⁴C]phenylalanyl-tRNA were present as indicated. Expt. 2 was carried out both in the absence and in the presence of tRNA^{Phe} with 20pmol of ribosomes and 25pmol of [¹⁴C]phenylalanyl-tRNA; poly(U) was present as indicated. Different EF 1 preparations were used in the two experiments.

Total [¹⁴ C]phenylalanine bound (pmol/100 pmol of ribosomes)							
Expt. 1	Ribosomes (pmol)	[¹⁴ C]Phenylalanyl-tRNA (pmol)	No inhibitor pmol	Modeccin		Ricin	
				pmol	Inhibition (%)	(pmol)	Inhibition (%)
	20	25	51.52	45.11	12	47.31	8
	10	25	66.62	47.13	29	49.42	26
	20	50	78.13	55.64	29	53.24	32
Expt. 2	tRNA ^{Phe}	Poly(U) (µg)					
			10.64	7.39	31	6.81	36
			3.91	1.83	53	1.67	57
			34.56	31.21	10	31.60	9
			31.24	22.97	26	21.35	32
	+	5	31.90	17.80	44	19.02	40

EF 1-dependent binding of phenylalanyl-tRNA to ribosomes. Inconsistencies in the past literature are presumably due to the presence of trace amounts of EF 2 in either the ribosomal or EF 1 preparations, leading, in the presence of GTP, to the formation of di- and tri-phenylalanine not measurable as hot-acid-insoluble radioactivity. Inhibition of their formation would have simulated an inhibition of the binding reaction assayed by the Millipore-filter technique, the degree of inhibition depending on the number of ribosomes active in binding EF 1 and on the availability of [^{14}C]phenylalanyl-tRNA and of other components of the binding reaction. As shown in Table 2 (Expt. 2), decreasing the amount of poly(U) present in the reaction mixtures strongly increased the apparent inhibition of the binding reaction by modeccin or ricin, probably by decreasing the number of active ribosomes and thus realizing the conditions favourable for the formation of oligopeptides. It is interesting to observe that tRNA^{Phc} was absent and very small amounts of poly(U) were present in the experiments from which Carrasco *et al.* (1975) and Fernandez-Puentes *et al.* (1976) drew the conclusion that ricin inhibits the EF 1-dependent binding of aminoacyl-tRNA.

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