ENZYMATIC INACTIVATION OF EUKARYOTIC RIBOSOMES BY THE POKEWEED ANTIVIRAL PROTEIN

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1. Introduction

The pokeweed antiviral protein (PAP) has been shown to be a potent inhibitor of both eukaryotic protein synthesis [1–3] and the transmission of a number of plant and animal viruses [4–6]. This protein specifically affects the larger ribosomal subunit and the ribosomal interactions of both elongation factors, EF 1 and EF 2 [1]. The observed stoichiometry of PAP inhibition of protein synthesis suggests that PAP enzymatically affects ribosomes [1–3].

In this communication we report that PAP binds very weakly to *Artemia salina* ribosomes and inactivates ribosomes by an unknown enzymatic mechanism. We also report that ribosomes bearing [^{14}C]Phe-tRNA in the acceptor site are resistant to inactivation by PAP. In contrast EF 2 fails to protect ribosomes from inactivation by PAP.

2. Materials and methods

A. salina ribosomes, supernatant enzyme fraction, PAP, and [^{14}C]Phe-tRNA were prepared as in [2]. The purification of *A. salina* EF 1 and EF 2 will be described elsewhere.

The iodination of PAP was accomplished by a modification of the method in [7] used for ricin. Reaction mixtures contained in 1.0 ml: 20 mM Tris–HCl, pH 7.5, 1.55 mg PAP, 0.2 mM Na^{125}I (5000 Ci/mol), 160 μg lactoperoxidase (Calbiochem) and total 35 μmol H_2O_2 added at 1 min intervals in 15 separate 10 μl aliquots at 25°C. 20 μmol 2-mercaptoethanol were added to the mixture which was then passed through Sephadex G-25 and the [^{125}I]PAP concentrated to approx. 1 mg/ml by ultrafiltration. [^{125}I]PAP prepared in this manner retained >90% of its inhibitory activity.

Polyphenylalanine synthesis was carried out in 0.25 ml reaction volumes containing 20 mM Tris–HCl, pH 7.5, 80 mM KCl, 4 mM $MgCl_2$, 2.5 mM DTT, 0.2 mM GTP, 40 pmol [^{14}C]Phe-tRNA (100 Ci/mol), 40 μg polyuridylic acid, 250 μg postribosomal supernatant enzyme fraction, and 200 μg washed ribosomes. Reactions were carried out for 5 min at 37°C and polyphenylalanine synthesis determined as in [2].

3. Results

3.1. *Binding of [^{125}I]PAP to ribosomes*

Centrifugation of ribosomes through a sucrose pad almost completely removes added [^{125}I]PAP (table 1). Under conditions which produce maximal inactivation of ribosomes less than 0.01 pmol [^{125}I]PAP is sedimented with the ribosomes (45 pmol). If centrifugation is performed in the absence of the sucrose pad to study equilibrium binding as accomplished [8] to study the binding of ricin less than 1% of [^{125}I]PAP added was found bound to the ribosomes (unpublished observation). The data demonstrate that PAP has a very low affinity for ribosomes and is readily removed by centrifugation.

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Table 1
Binding of [^{125}I]PAP to ribosomes

[^{125}I]PAP added (pmol)	[^{125}I]PAP bound (pmol)
1.0	<0.01
3.0	<0.01
10.0	0.03
30.0	0.08

The indicated amounts of [^{125}I]PAP (6940 cpm/pmol) were incubated in 0.25 ml vol. containing 20 mM Tris–HCl, pH 7.5, 80 mM KCl, 4 mM $MgCl_2$, 2.5 mM DTT and 200 μg ribosomes for 5 min at 37°C. The samples were layered over 1 ml pads of 10% sucrose containing the same salt concentrations as the samples and centrifuged at 40 000 rev./min for 30 min in a Beckman type 40 rotor. The supernatant was removed by aspiration, the pellets dissolved in 0.25 ml water, and radioactivity determined by liquid scintillation

3.2. *Polyphenylalanine synthesis upon ribosomes after removal of PAP*

Ribosomes were incubated with different concentrations of PAP, isolated by centrifugation, and tested for the ability to synthesize polyphenylalanine. Increasing concentrations of PAP reduced the ribosomal activity, maximal inhibition being obtained with 10 pmol PAP (fig.1). Such a result directly demonstrates the enzymatic action of PAP upon ribosomes.

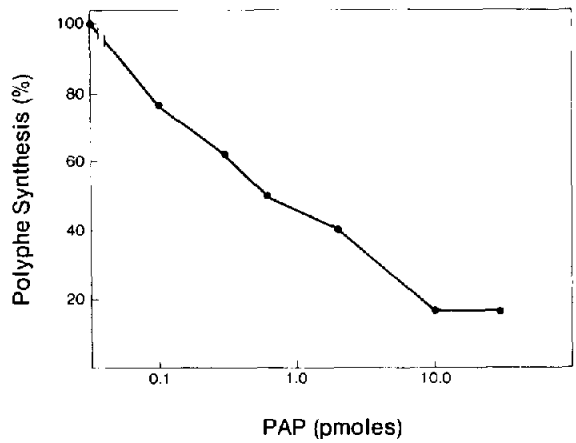

Fig.1. Polyphenylalanine synthesis upon ribosomes isolated after treatment with PAP. Ribosomes were incubated with the indicated amounts of PAP and isolated as described in table 1 legend. Pellets were dissolved directly in the polyphenylalanine synthesis buffer and assayed as indicated in section 2. Uninhibited polyphenylalanine synthesis was 21.9 pmol phenylalanine.

3.3. *Protection of ribosomes from PAP inactivation*

Since PAP affects elongation factor interaction with the ribosome it was of interest to ascertain if elongation factor binding influenced the action of PAP. [^{14}C]Phe-tRNA was bound to ribosomes with EF 1 and treated with various concentrations of PAP. Ribosomes isolated after treatment retained full activity in polyphenylalanine synthesis (table 2). To test the effect of PAP upon acceptor site ribosomes in the absence of EF 1 [^{14}C]Phe-tRNA was non-enzymatically bound and the ribosomes tested for inactivation after isolation. Ribosomes bearing acceptor site [^{14}C]Phe-tRNA were found to be resistant to low concentrations of PAP but were partially inactivated at the highest concentration tested (table 2). The results indicate that acceptor site ribosomes are partially resistant to PAP inactivation and that the presence of EF 1 completely protects the ribosomes.

The effect of EF 2 upon PAP inactivation was tested with ribosomes bearing preformed EF 2·GDP complex. Ribosomes bearing complex were incubated with PAP at 0°C. The isolated ribosomes were found to be inactivated readily by PAP (table 2) nearly to the extent observed with empty ribosomes (fig.1). The results demonstrate that EF 2 has little protective effect against the action of PAP and PAP acts efficiently at 0°C.

4. Discussion

The failure of PAP to appreciably bind to ribosomes and the isolation of ribosomes which remain inactive in the absence of PAP demonstrate that PAP affects ribosomes in an enzymatic manner. Such an enzymatic action is similar to that observed with the A-chains of the toxins, ricin and abrin [9]. A major difference between PAP and the ricin A-chain is that PAP binding to ribosomes is a much weaker interaction than that reported for the ricin A-chain [8].

The protection afforded by acceptor site ribosomes in presence of EF 1 and lack of protection by EF 2 is in contrast to the reported protection of ribosomes from ricin and abrin action by EF 2 and

Table 2
The effect of elongation factor interaction and acceptor site Phe-tRNA upon PAP inactivation of ribosomes

PAP	Polyphenylalanine Synthesis					
	A-site	(enzymatic)	A-site	(nonenzymatic)	EF 2·GDP complex	
(pmol)	(pmol)	(%)	(pmol)	(%)	(pmol)	(%)
0	14.5	100	24.3	100	21.6	100
0.3	13.6	94	29.4	121	17.3	80
3.0	15.9	110	19.9	82	10.8	50
30.0	18.1	125	10.6	44	5.9	27

Enzymatic A-site ribosomes were prepared in 0.2 ml reaction volumes containing 20 mM Tris–HCl, pH 7.5, 120 mM KCl, 8 mM $MgCl_2$, 0.2 mM GTP, 40 μg polyuridylic acid, 200 μg ribosomes, 20 pmol [^{14}C]Phe-tRNA and 16.5 μg EF 1 by incubation at 25°C for 20 min. The amount of acceptor site binding was determined to be 6.6 pmol [^{14}C]Phe-tRNA. Nonenzymatic A-site ribosomes were prepared in 0.1 ml reaction mixtures in which the $MgCl_2$ concentration was raised to 20 mM and EF 1 and GTP were omitted. The mixtures were diluted to final vol. 0.2 ml to adjust the solution to 10 mM $MgCl_2$ prior to PAP treatment. Complexes of EF 2·GDP on ribosomes were prepared in 0.2 ml vol. containing 20 mM Tris–HCl, pH 7.5, 80 mM KCl, 4 mM $MgCl_2$, 2.5 mM DTT, 0.2 mM GTP, 200 μg ribosomes, and 40 μg EF 2 by incubation at 37°C for 5 min. The extent of complex formation was determined to be 3.8 pmol with [^{14}C]GTP (50 Ci/mol). All mixtures were treated with PAP, isolated, and assayed as in fig.1 except that ribosomes bearing EF 2·GDP complex were incubated with PAP at 0°C to reduce the turn over of the complex

the failure of acceptor site Phe-tRNA and EF 1 to protect the ribosomes [10]. Other results have lead to the suggestion that possibly acceptor site ribosomes are resistant to PAP, ricin and abrin [11]. This suggestion is confirmed, at least for PAP, by our results.

The observed protection of acceptor site ribosomes from PAP inactivation is an indication that the enzymatic action of PAP occurs near or at this site involved in the interaction of both elongation factors. The results also suggest that PAP may act upon the ribosome in a manner distinct from that of the A-chains of ricin and abrin.

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