A CYTOKININ BINDING PROTEIN FROM HIGHER PLANT RIBOSOMES

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Received April 2,1975

SUMMARY

Two types of cytokinin binding sites exist on higher plant ribosomes. One of these, a high affinity site, binds cytokinins at low concentrations, is saturated at one cytokinin molecule per ribosome, is specific for substances with cytokinin activity, and is readily washed from the ribosome with 0.5 M KCl. The binding moiety has the characteristics of a protein and binds cytokinins apart from the ribosome.

INTRODUCTION

Berridge et al. (1,2) described reversible binding of cytokinins to 83S plant ribosomes and reported that pretreatment of the ribosomes with 0.5 M NH_4Cl did not decrease the extent of binding. These workers concluded that several cytokinin binding sites exist per ribosome; they were unable to saturate the sites or to determine the total number because of the limited solubility of the cytokinins used. Because of the potential significance of these observations, we have reexamined the interaction of cytokinins with ribosomes and confirm the binding. We find, however, evidence for at least two types of cytokinin binding sites on higher plant ribosomes and describe cytokinin interactions with each. In addition we report here on the nature of a cytokinin binding moiety which can be washed from the ribosomes.

METHODS AND MATERIALS

Ribosome preparation Freshly milled wheat germ (Dixie-Portland Mills, Arkansas City, Kansas) was ground at 4° in a mortar and pestle with 10% w/w insoluble polyvinylpyrrolidone and 150 ml/100 gm wheat germ of the following buffer: 25mM tris acetate pH7.6, 5mM magnesium acetate, 5mM β-mercaptoethanol, 25 mM potassium

acetate, 0.1M sucrose (ribonuclease free). The homogenate was centrifuged at 30,000 x G for 15 minutes, the pellet reground in the same volume of buffer and again centrifuged. The combined supernatants were centrifuged at 150,000 x G for 3 hours. The ribosomal pellet was resuspended in the buffer minus sucrose, centrifuged at 30,000 x G for 15 minutes to remove debris and pelleted a second time. The average final yield of ribosomes was $100A_{260}$ units/ gm wheat germ. Although Berridge et al.(1,2) assumed a ribosome molecular weight of 2.7×10^6 daltons, the accepted figure is nearer 4.0×10^6 daltons (3) for higher plant ribosomes. If one assumes an A_{260} of 16 (1) for 1 mg/ml of ribosomes, then 1 A_{260} is equivalent to 15.625 p moles. Ribosomes isolated in this manner were highly active in poly U directed polyphenylalanine synthesis in an in vitro system. Ribosomes were prepared from tobacco callus cultures and rat liver by minor modifications of the procedures described above. Escherichia coli ribosomes were prepared as previously described (4).

Binding studies Cytokinin binding was determined by a centrifugation technique or by equilibrium dialysis. In each case the standard buffer contained 25 mM tris acetate pH 7.6, 5mM magnesium acetate, 60 mM potassium acetate, and 5 mM β-mercaptoethanol. In the centrifugation studies an amount of ribosomes generally greater than 75 A_{260} units were suspended in 2 ml of the standard buffer at 0° with additions as noted. The ribosomes were pelleted by centrifuging at 150,000 X G for 3 hours. The supernatant was decanted and the centrifuge tube carefully drained before the ribosomes were resuspended in two successive 100 µl aliquots of water. These were combined and added to 10 ml Bray's solution or Aquasol (New England Nuclear) for estimation of radioactivity by liquid scintillation spectrometry. No attempt was made to correct for radioactivity trapped in the ribosome pellet since the results were in good agreement with those obtained by equilibrium dialysis suggesting that the trapped volume must be a very small fraction of the total.

Equilibrium dialysis was carried out at 4° for periods from 18 to 50 hours although preliminary work established that equilibration was achieved within 12 hours. In the majority of experiments 100 A_{260} units of ribosomes in 1.0 ml of

the standard buffer used for centrifugation studies was dialyzed against 14 ml of the same buffer containing additions as noted. In some cases much higher amounts of ribosomes or much larger volumes of dialysis buffer were used.

RESULTS AND DISCUSSION

Effect of Cytokinin Concentration on Binding That substantial cytokinin binding to higher plant ribosomes does occur is shown in a typical equilibrium dialysis study (Table 1). Ribosome preparations from rat liver or E. coli bound substantially smaller amounts of the cytokinin 6-benzylaminopurine (BA) than did higher plant ribosomes (Table 1). Changes in magnesium concentration over the range 1 to 20 mM had no effect on the extent of binding which occurred at 6.76 μM BA although the

Table I

CYTOKININ BINDING TO RIBOSOMES

Preparation	DPM per 0.1 ml at equilibration (24 hr dialysis)	Moles Cytokinin Bound per Mole Ribosomes
1. Buffer external to dialysis membrane	13,339	———
2. Control buffer internal to dialysis membrane	13,150	———
3. Wheat germ ribosomes 400 A_{260}/ml	35,132	1.24
4. Tobacco ribosomes 216 A_{260}/ml	34,832	2.26
5. Rat liver ribosomes 80 A_{260}/ml	14,344	0.34
6. E. coli ribosomes 400 A_{260}/ml	18,785	0.20

0.5 ml of each preparation was dialyzed at 4° against 100 ml of standard dialysis buffer, 4.7 μM in [8-^{14}C] 6 - Benzylaminopurine sp. act. 12.8 mCi/mm (Amersham-Searle). Aliquots of the ribosome preparations inside the dialysis bag were counted in Bray's solution. (See Methods and Materials for details of counting and calculation of ribosome molarities.)

binding was reduced about 10% both in the absence of added magnesium in the buffer and at 50 mM magnesium.

Number and Type of Binding Sites In order to look more closely at the characteristics of the binding, a series of equilibrium dialysis studies were performed in which relatively large amounts of ribosomes were dialyzed against increasing concentrations of [^{14}C] BA at decreasing specific activities. The results plotted by the method of Scatchard et al. (5) yielded a biphasic curve (Fig 1,A) indicative of two types of binding sites. Scatchard plots of this type have been inter-

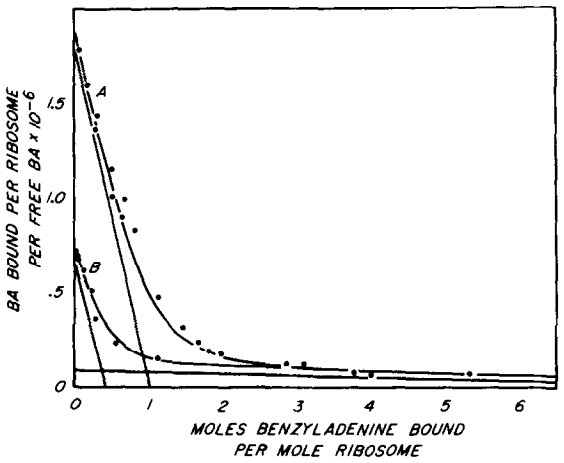

Fig 1. Scatchard plot of concentration dependent binding of 6-Benzylaminopurine to native ribosomes (A) and 0.5 M KCl washed ribosomes (B). Experimental points were obtained by dialyzing aliquots of ribosomes (1 ml containing 100 A_{260}) against 15 ml of dialysis buffer containing [8-^{14}C] BA at concentrations from 2.5 X 10^{-8}M to 7.5 X 10^{-5}M at specific activities from 12.8 mCi/mm to 0.013 mCi/mm. At equilibrium (25 hr.) radioactivity internal and external to the dialysis bag was estimated and binding was calculated as described in Methods and Materials. The slope and position of the straight lines were calculated from the experimental data by adaptations of the method of Hunston(8). The curved lines are graphical sums of the two straight lines.

preted (e.g. 6,7) to indicate a limited number of binding sites of high affinity together with a larger number of low affinity binding sites. The apparent intercept of the high affinity site indicates a single cytokinin binding site on the ribosome together with multiple low affinity sites. The binding constant of the high affinity site has been calculated to be 1.6×10^6 M^{-1} and the low affinity 9.0×10^3 M^{-1}. A similar concentration dependent binding study was performed by the centrifugation technique with nearly identical results.

Ribosomes washed in 0.5 M KCl bind substantially less BA than untreated ribosomes when dialyzed against low BA concentrations (Table II). Material washed

TABLE II

CYTOKININ BINDING TO KCl AND NH_4Cl TREATED RIBOSOMES AND RIBOSOME WASHES

	Preparation	DPM per 0.1 ml After 24 Hr Dialysis	DPM/mg Protein	Moles Cytokinin Bound per mole Ribosomes
1.	Buffer external to dialysis membrane	10,853	———	———
2.	Control buffer internal to dialysis membrane	10,890	———	———
3.	Control Ribosomes 230 A_{260}/ml	19,979	———	.90
4.	0.5 M KCl washed Ribosomes 384 A_{260}/ml	12,891	———	.12
5.	Dialyzed KCl wash 2.4 mg protein/ml	24,285	55,967	———
6.	0.2 M NH_4Cl washed Ribosomes 385 A_{260}/ml	23,648	———	.76
7.	Dialyzed NH_4Cl wash 2.9 mg protein/ml	16,011	17,786	———

385 A_{260} Ribosomes were suspended at 0° in 2 ml of a buffer 25 mM tris-acetate pH 7.5, 5 mM magnesium acetate, 5 mM β-mercaptoethanol and either 0.5 M KCl or 0.2M NH_4Cl and immediately pelleted by centrifugation (2.5 hr. at 105,000 x G). The supernatant was decanted and dialyzed 24 hr. against standard dialysis buffer (Methods and Materials). The ribosome pellet was resuspended in 1 ml dialysis buffer. Dialysis of the ribosomes and supernatants was performed at 4° for 18 hr against 100 ml dialysis buffer 5.3 μM in [8-^{14}C] BA sp. act, 12.8 mCi/mm.

off the ribosome by this treatment and subsequently dialyzed to remove KCl effectively binds cytokinins by itself (Table II). A Scatchard plot of concentration dependent cytokinin binding to KCl treated ribosomes (Fig 1,B) reveals quite clearly that most of the high affinity sites have been removed by the KCl treatment without affecting the low affinity sites. This dual nature of cytokinin binding to ribosomes was overlooked by Berridge et al. (1,2) apparently because at the cytokinin concentration used in their studies (2.3×10^{-5}M) much of the binding would have been to the low affinity site. A large number of cytokinins, cytokinin analogs and purine derivatives have been tested for their ability to compete with BA for the high affinity binding site. Although this data will be presented in detail elsewhere, we find a direct relationship between cytokinin

Table III

EFFECT OF ENZYMES AND HEAT ON ABILITY OF KCl WASH OF RIBOSOMES TO BIND CYTOKININ

	Treatment	DPM per 0.25 ml after 40 hr dialysis
1.	Buffer external to dialysis membrane	4,804
2.	KCl wash of ribosomes (2.4 mg protein ml) incubated at 37° for 30 minutes.	23,535
3.	As # 2 plus 2 mg/ml trypsin (Sigma Type IX)	4,980
4.	Buffer plus 2 mg/ml trypsin incubated as #2	4,926
5.	As #2 plus 2 mg/ml ribonuclease (Sigma type 1A)	20,884
6.	Buffer plus 2 mg/ml ribonuclease incubated as #2	5,022
7.	KCl wash incubated at 100° for 30 minutes	5,719

The KCl ribosome wash was dialyzed against a large excess of standard dialysis buffer for 24 hr before being used in this study. The incubations were performed in dialysis buffer and the preparation was then dialyzed 40 hours at 4° against 100 ml dialysis buffer 6.76×10^{-7}M in [8-^{14}C] BA sp. act. 12.8 mCi/mm.

activity and ability to compete with BA for binding to the high affinity site.

Nature of the Binding Moiety That the binding substance removed from the ribosomes by 0.5 M KCl is a protein may be seen in Table III. Both boiling and trypsin digestion abolished the ability of the extract to bind cytokinin while ribonuclease treatment had little effect. The binding protein may itself exist in an equilibrium, free in the cytosol or bound to the ribosome, since preliminary evidence indicates that a similar cytokinin binding substance may be isolated from the post-ribosomal supernatant. The crude 150,000 X G supernatant dialyzed against dialysis buffer 5.3 μM in [8-^{14}C] BA sp. act. 12.8 mCi/mm. bound more than 10^4 DPM per mg protein in excess of the control. Isolation and purification of the cytokinin binding protein will be reported elsewhere.

This work was supported by NSF grant BMS74-07386 and by Biomedical Sciences Support Grant RR-07037 to JEF. In addition JLE was supported by a University of Kansas General Research Grant.

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