

Influence of Spermine on Amino Acid Incorporation by Free, Bound, and Reattached Ribosomes from Rat Liver

JAHANGIR A. KHAWAJA

Department of Medical Chemistry, University of Helsinki, SF-00170 Helsinki 17, Finland

1. Free ribosomes were attached to endoplasmic reticulum membranes *in vitro* in the presence of Mg^{2+} or spermine (reattached ribosomes). Reattached ribosomes were active in endogenous and in poly U-directed phenylalanine incorporation into polypeptides. The abilities of various ribosomal preparations to incorporate amino acids in the presence and absence of polyuridylic acid (Poly U) were compared.

2. The effects of exogenous spermine on amino acid incorporation by free and bound ribosomes were compared in order to examine whether the polyamine had any preferential effect on one or the other class of ribosomes.

3. Poly U stimulated [^{14}C]phenylalanine incorporation significantly more with free ribosomes than with either bound or reattached ribosomes.

4. At a suboptimum Mg^{2+} concentration exogenous spermine (0.3 mM) substantially stimulated poly U-directed phenylalanine incorporation. Spermine stimulated amino acid incorporation to similar extents with free and bound ribosomes. Bound ribosomes were relatively more resistant to inhibitors of protein synthesis such as cycloheximide and sodium fluoride.

Within the cytoplasm of most animal cells, a fraction of ribosomes is topographically segregated from the rest of the ribosomal population by attachment to membranes.^{1,2} It is only recently that factors involved in the attachment of ribosomes to endoplasmic reticulum membranes are beginning to be more clearly understood.³⁻⁵ Previous work from this laboratory has suggested that naturally occurring polyamines, such as spermine, may play a role in the ribosome-membrane interactions.⁶⁻⁸

The polyamines spermine and spermidine are associated with isolated bacterial⁸⁻¹¹ and mammalian ribosomes.^{7,12-15} Studies on the effect of polyamines on various amino acid incorporation systems have mainly been concerned with bacteria.¹⁶⁻²⁰ Depending on the concentration of the polyamine

in the system, spermine was able to either stimulate or inhibit cell-free protein synthesis.¹⁹ Relatively less data are available on the effect of polyamines on protein synthesis in systems derived from mammalian tissues. Polyamines have been shown to affect protein synthesis in cell-free systems from mouse tumour cells,²¹ from rat liver,²² and with cerebral cortex ribosomes.²³ Polyamines stimulated protein synthesis at suboptimum Mg^{2+} concentrations, but inhibited it at optimum or higher concentrations of Mg^{2+} in the incubation mixture.²² However, the precise mode of action of polyamines in affecting protein synthesis is not yet fully understood.

The aim of the present investigation was to study the influence of the polyamine spermine on the protein synthesizing activities of free, bound, and reattached ribosomes, in order to see whether spermine had any preferential effect on one or the other class of ribosomes.

MATERIAL AND METHODS

Puromycin, cycloheximide (Acti-dione), poly U, ATP, GTP, creatine phosphate and creatine phosphokinase (ATP creatine phosphotransferase, E.C. 2.7.3.2) were purchased from Sigma Chemical Company, U.S.A. Sodium fluoride was a product of E. Merck (Darmstadt). L-3-[¹⁴C]Phenylalanine (513 mCi/mmol) was supplied by the Radiochemical Centre, Amersham, Bucks, England. [³H]Poly U (8.1 mCi/mmol) was purchased from Schwarz BioResearch, Inc., U.S.A. Dithiothreitol was purchased from Calbiochem, U.S.A. Solutions of ATP and GTP were adjusted to pH 7.5 with KOH. The source of the rest of the material and the animals used in the present study have been described previously.⁶⁻⁸

Preparation of subcellular fractions

Free and bound ribosomes were prepared as described previously,^{6,7} except that 3 mM glutathione (reduced form, Sigma) was included in the isolation medium. Microsomes were prepared by centrifugation of postmitochondrial supernatant fraction at 105 000 *g* (av.) for 1 h and washed once in the isolation medium. Sucrose gradient analysis showed that, in addition to bound ribosomes, this preparation also contained free ribosomes. Preparation of total endoplasmic reticulum membranes has been described.⁸

Preparation of reattached ribosomes. Free ribosomes (1.2 mg RNA) and membranes (6 mg protein) were incubated either in Tris-KCl-MgCl₂ (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂), or Tris-KCl + 0.5 mM spermine at 0°C for 60 min in a total volume of 2 ml. The mixture was then loaded onto a discontinuous gradient, consisting of 1 ml of 1.31 M sucrose overlaid on 2 ml of 2 M sucrose, all made in the buffer having the same ionic composition as the one used for incubation. The gradient was centrifuged in the SW 50.1 rotor of a Spinco L 50 centrifuge for 20 h at 149 000 *g* (av.) at 4°C. In some experiments the gradient was modified for the angle rotor 50 Ti, which gave similar results but a greater yield of reattached ribosomes. After the completion of centrifugation the interphase layer containing membranes and reattached ribosomes was collected, diluted with the incubation buffer, and centrifuged at 68 000 *g* for 3 h to obtain a pellet. The pellet was gently suspended in either Tris-KCl-MgCl₂ or Tris-KCl + 0.3 mM spermine, as appropriate and the suspension was finally centrifuged at 68 000 *g*.

The term reattached ribosomes is used for convenience to denote isolated free ribosomes attached *in vitro* to membranes. It is not meant to indicate that free ribosomes were necessarily bound to membranes *in vivo*.

Preparation of pH 5 enzyme fraction. This was prepared from rat liver by a modification of the method described by Hoagland *et al.*²⁴ The 105 000 *g* supernatant was diluted with 2 vol. of Tris-KCl-MgCl₂ buffer containing 3 mM glutathione. The pH was adjusted to 5.1 by dropwise addition of 1 M acetic acid with constant stirring. The precipitate was suspended in Tris-KCl-MgCl₂ and the pH was adjusted back to 7.5. The suspension

was centrifuged at 3000 *g* for 3 min to remove insoluble material followed by dialysis against Tris-KCl-MgCl₂ for 12 h. The fraction was then centrifuged for 3 h at 149 000 *g* to remove any monosomes present. The clear suspension was divided in small portions and stored frozen at -70°C until used.

Isolation of partially purified aminoacyl transferase I and II ("PTF" fraction). The 40–70 % ammonium sulphate fraction which contains the aminoacyl transferase I and II was prepared from the cell sap remaining after precipitation of the pH 5 fraction.²⁵ The pH of the supernatant was adjusted to 6.9–7.0 with 2 M KHCO₃ and sufficient (NH₄)₂SO₄ was added to give 40 % saturation. The resulting suspension was centrifuged at 10 000 *g* for 10 min. The supernatant obtained was adjusted to 70 % with respect to (NH₄)₂SO₄ and centrifuged again at 10 000 *g* for 10 min. The precipitate was suspended in Tris-KCl-MgCl₂ containing 3 mM glutathione and dialyzed against the same buffer for 8–12 h.

Assay of amino acid incorporation. The standard cell-free system for amino acid incorporation²⁶ (0.5 ml) contained: 50 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 80 mM NH₄Cl, 1 mM dithiothreitol, 10 mM creatine phosphate, 1 mM ATP, 0.4 mM GTP, 19 unlabelled L-amino acids at 0.5 mM, 0.5 μCi of [¹⁴C]phenylalanine, 5 μg of phosphokinase, 1 mg of pH 5 enzyme protein, 0.7 mg PTF fraction protein and 100 μg ribosomes (given as RNA). Poly U (100 μg) was present where indicated. Ribosomes, pH 5 enzyme, and PTF fraction contributed Mg²⁺ to a final concentration of 2 mM. The amounts of pH 5 enzyme and PTF fraction were experimentally found to be optimal for promoting [¹⁴C]phenylalanine incorporation into polypeptide. The incubation medium described above is referred to as incubation medium I. In some experiments, Mg²⁺ was reduced to a final concentration of 2 mM and spermine was added at 0.3 mM. This was designated as incubation medium II.

Determination of protein radioactivity. Following incubation at 37°C for periods up to 60 min, the reaction was stopped by addition of an equal volume of cold 10 % (w/v) trichloroacetic acid solution containing 0.1 % unlabelled phenylalanine. The mixture was heated at 90°C for 20 min to extract RNA. The precipitate was washed three times with 5 % trichloroacetic acid, dissolved in 0.5 N NaOH and further processed for the determination of radioactivity as described previously.⁶ The counts obtained by incubation of the complete system minus ribosomes were subtracted from the counts obtained when the complete system was used.

Determinations of RNA and protein have been described.⁶

RESULTS

Amino acid incorporation by free and bound ribosomes. The distribution of RNA and protein in different isolated fractions is shown in Table 1. The RNA/protein ratio of reattached ribosomes, prepared under the present ex-

Table 1. RNA/protein ratio of various ribosomal and membrane preparations. The fractions were prepared as described in the Material and Methods section. Bound ribosomes were also carefully purified from any contaminating lysosomes (see text). The results are expressed as the mean ± standard deviation (number of experiments given in parentheses).

Fraction	Ratio RNA/protein
Total endoplasmic reticulum membranes	0.01 ± 0.006 (35)
Free ribosomes	0.61 ± 0.090 (29)
Bound ribosomes	0.04 ± 0.008 (19)
Bound ribosomes (purified)	0.06 (2)
Reattached ribosomes (Tris-KCl-MgCl ₂)	0.05 ± 0.006 (5)
Reattached ribosomes (Tris-KCl + spermine)	0.05 ± 0.020 (4)

perimental conditions, was close to that of authentic bound ribosomes. It was found that repeated washing of the reattached ribosomes with Tris-KCl-MgCl₂ did not reduce their RNA content.

Incorporation of [¹⁴C]phenylalanine into both free and bound ribosomes was similar with respect to dependence upon the addition of ribosomes, an energy generating system, pH 5 enzyme, and PTF fraction. Omission of the unlabelled amino acid mixture did not affect the poly U-directed [¹⁴C]phenylalanine incorporation into free and bound ribosomes to any considerable extent. When various ribosomal preparations were tested for amino acid incorporation using medium I, free ribosomes were found to be more active than bound ones, but the microsome fraction was the most active (Table 2). The incorporation of phenylalanine was enhanced 3-fold by the addition of poly U to the free ribosome system. The stimulation by poly U was significantly less (1.5-fold) in the bound ribosome system. It can be seen from Table 2 that free ribosomes after their attachment to membranes (reattached ribosomes) became less responsive to stimulation by poly U. The relative stimulation was only 2.4-fold and 2.2-fold in reattached ribosomes prepared in Tris-KCl-MgCl₂ and Tris-KCl + 0.5 mM spermine, respectively. In the latter case, the incubation was carried out in medium II (containing 2 mM MgCl₂ and 0.3 mM spermine). It can also be seen from Table 2 that the protein synthesizing activity of the microsome fraction which contained free ribosomes in addition to those bound to membranes (*cf.* Ref. 27) was stimulated to an intermediate value between those obtained with systems containing either free or bound

Table 2. Stimulation by poly U of the incorporation of [¹⁴C]phenylalanine into polypeptide by free, bound and reattached ribosomes.

The fractions were prepared as described in the Material and Methods section. In No. 3 bound ribosomes were purified free from lysosomes (see text). Incubation medium I (containing 10 mM Mg²⁺) was used with fractions 1–5, whereas incubation medium II (containing 2 mM MgCl₂ + 0.3 mM spermine) was employed with fraction No. 6. Incubation was at 37°C for 30 min. Details of incubation and determination of radioactivity in hot trichloroacetic acid-insoluble material are described in the Material and Methods section.

Fraction	[¹⁴ C] Phenylalanine incorporated Counts/min × 10 ⁻³ per mg RNA		
	No poly U added	100 μg poly U added	Relative stimulation by poly U
1 Free ribosomes	44.1	141.6	3.2
2 Bound ribosomes	36.1	56.5	1.5
3 Bound ribosomes (purified) ^a	40.3(31.0)	81.9(58.2)	2.0
4 Microsomes	65.7	176.1	2.7
5 Reattached ribosomes (Tris-KCl-MgCl ₂)	36.0	88.2	2.4
6 Reattached ribosomes (Tris-KCl + spermine)	46.9	105.8	2.2

^a A different preparation from that used in No. 2. The figures in parentheses show the incorporation before purification.

ribosomes. The difference in response of free and bound ribosomes with respect to stimulation by poly U was a consistent finding.

The different behaviour of free and bound ribosomes could have been due to contamination of the latter with lysosomes.²⁸ To test this possibility, bound ribosomes were purified to remove the lysosomal contamination.²⁸ In the cell-free system, they gave results essentially similar to those described above, although the protein synthesizing activity of bound ribosome fraction was slightly increased (Table 2).

Effect of spermine on poly U-directed [¹⁴C]phenylalanine incorporation into free and bound ribosomes. Because polyamines can partially replace Mg²⁺ in cell-free protein synthesis,^{18,19,22} the optimum concentration of Mg²⁺ for the present system was established. Both free and bound ribosomes required an optimum Mg²⁺ concentration of about 8 mM. It should be pointed out that various subcellular fractions (ribosomes, pH 5 enzymes, and PTF fractions) contributed 1 μmol of Mg²⁺ to the incubation mixture so that the final concentration of Mg²⁺ was higher than that indicated in Fig. 1. The free Mg²⁺ concentration in the system cannot be accurately stated owing to the difficulty in assessing the extent of Mg²⁺ interactions with anionic groups on ATP, GTP, RNA, phosphate *etc.*²⁹

At all concentrations of the polyamine tested, phenylalanine incorporation was not stimulated at optimum Mg²⁺ concentration (Fig. 1). Instead, addition of more than 0.025 mM spermine reduced the amino acid incorporation. Incorporation was about 1/6 of control levels at 0.3 mM spermine. This is compar-

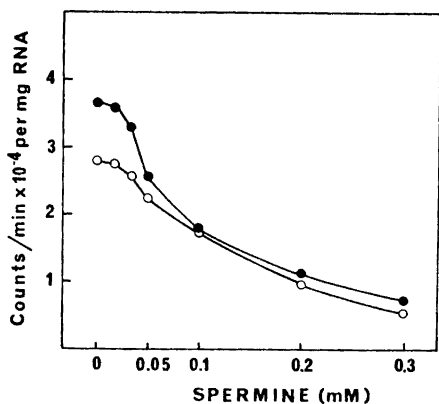


Fig. 1. Effect of exogenous spermine on poly U-directed [¹⁴C]phenylalanine incorporation into hot trichloroacetic acid-insoluble material at optimum Mg²⁺ concentration (final Mg²⁺ concentration: 10 mM). Spermine was added directly to the assay medium. Incubation was for 30 min. Protein radioactivity was determined as described in Material and Methods section. Free ribosomes, ●; bound ribosomes, ○.

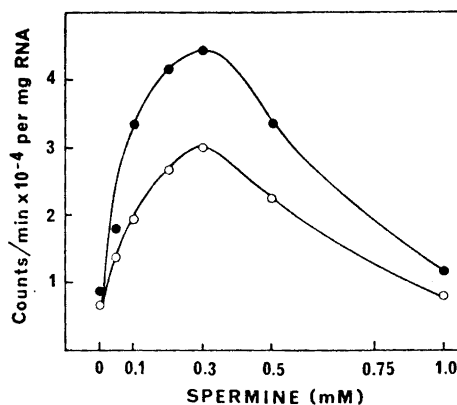


Fig. 2. Effect of exogenous spermine on the [¹⁴C]phenylalanine incorporation into hot trichloroacetic acid-insoluble material by free and bound ribosomes at suboptimum (final Mg²⁺ concentration: 2 mM) Mg²⁺ concentration. Conditions of incubation *etc.* are identical to those given for Fig. 1.

able to the results obtained with bacterial and total mammalian ribosomes.^{19,2} The inhibition of protein synthesis at higher concentrations of the polyamine is probably due to the resulting excessive aggregation of ribosomes (*cf.* Refs. 19, 22).

Fig. 2 shows the effect of different concentrations of spermine on the poly U-directed phenylalanine incorporation at suboptimum Mg^{2+} concentration. The incubation buffer did not contain any $MgCl_2$ but the various fractions contributed Mg^{2+} to a final concentration of 2 mM. Incorporation of [¹⁴C] phenylalanine was maximum at a spermine concentration of 0.3 mM, when [¹⁴C]phenylalanine incorporation was stimulated about 5-fold (Fig. 2). Increasing the spermine concentration from 0.3 mM to 1 mM resulted in a reduction of the amino acid incorporation with a negligible stimulation with 1 mM spermine.

To find out whether spermine effects the kinetics of amino acid incorporation in various systems, endogenous and poly U-directed phenylalanine incorporation were studied as a function of time using either free or bound ribosomes in the presence and absence of spermine. Poly U-directed incorporation of phenylalanine was linear for about 30 min with both free and bound ribosomes. In the absence of exogenous messenger a plateau was reached in about 15 min. Essentially similar results were obtained with free and bound ribosomes in the cell-free systems containing 0.3 mM spermine and 2 mM Mg^{2+} .

To examine whether the lower stimulation of bound and reattached ribosomes was due to degradation of poly U by lysosomal contamination of these fractions, the degradation of [³H]-poly U by free ribosomes, bound ribosomes and membranes was studied (Table 3). There was no significant difference in

Table 3. Degradation of [³H]poly U in the systems containing free or bound ribosomes in the presence and absence of spermine. In the first experiment, the degradation of poly U was followed by incubating 25 nCi of [³H]poly U in a medium containing 10 mM Tris-HCl (pH 7.6), 8 mM Mg_2Cl , 80 mM NH_4Cl , 1 mM DTT, ribosomes (100 μ g RNA) or total endoplasmic reticulum membranes (1.6 mg protein). Experiment 2 was similar to experiment 1 except that spermine was added to the incubation medium at 0.3 mM and Mg^{2+} was present at a concentration of 2 mM. The percentage degradation of poly U is based on trichloroacetic acid-soluble radioactivity.

	[³ H]Poly U hydrolysed (%)	
	Expt. 1	Expt. 2
Free ribosomes	2.7	3.0
Bound ribosomes	6.9	6.4
Membranes	6.5	6.4

the extent of poly U degradation by the three fractions. Poly U degradation was also unaffected by 0.3 mM spermine.

Susceptibility of free and bound ribosomes to inhibitors of protein synthesis. To explain the observed behavioural differences between the free and bound

Table 4. Effect of different inhibitors of protein synthesis on the poly U-directed [^{14}C]-phenylalanine incorporation by free and bound ribosomes. Incubation medium I was composed of all constituents of the standard incubation mixture described in the Material and Methods section. The final concentrations of inhibitors were: cycloheximide, 1.63 mM; puromycin, 1 mM and NaF, 20 mM. The time of incubation was 15 min. The results are expressed as the mean of three experiments (range in parentheses).

Fraction	% inhibition of [^{14}C]phenylalanine incorporation compared with the control:		
	Cycloheximide	Puromycin	NaF
Free ribosomes	70 (65–84)	65 (58–72)	75 (74–76)
Bound ribosomes	41 (26–50)	60 (58–62)	52 (48–57)

ribosomes, the effects of several inhibitors of protein synthesis were tested. Bound ribosomes were consistently more resistant than free ribosomes to cycloheximide and NaF, tested at different concentrations (Table 4).

DISCUSSION

Free ribosomes incorporated more amino acid than bound ribosomes both with and without exogenous messenger. This finding is in agreement with some reports,^{30–32} but differs from others.^{27,33–36} The composition of the isolation media used (*e.g.* amount of KCl),²⁷ the possible redistribution of ribosomes during preparation,³⁷ and the degree of contamination may explain the discrepancies in the reported results. Addition of poly U stimulated phenylalanine incorporation into free ribosomes more than into bound ribosomes. The greater response of free ribosomes to stimulation by poly U could be due to a partial loss of endogenous messenger, although most free ribosomes sediment in the polysomal region of the sucrose gradient (*cf.* Refs. 6, 7). On the other hand, it is possible that the smaller stimulation in bound ribosomes is due to the environmental influence of membrane-binding. The latter possibility is supported by the observation that the response of free ribosomes to stimulation by poly U was considerably reduced after their attachment to membranes *in vitro*. Degradation of poly U by bound or reattached ribosomes did not seem to be responsible for their lower response, as shown by the results presented in Table 3. When bound ribosomes were purified to remove any lysosomes present, the poly U-stimulation of phenylalanine incorporation was still greater with free, than with bound ribosomes.

The effect of polyamines on various amino acid incorporating systems has previously been reported,^{16–23} although the data have been mainly concerned with bacterial systems. Polyamines have been shown to stimulate the binding of aminoacyl-tRNA to yeast ribosomes³⁸ and the binding of poly U to *Escherichia coli* ribosomes.³⁹ They could partially replace Mg^{2+} in cell-free protein synthesis in both bacterial¹⁹ and mammalian systems.²² In the present study a spermine concentration of 0.3 mM gave maximum stimulation of amino acid incorporation provided the Mg^{2+} concentration was kept at a suboptimum level. In the presence of an optimum concentration of Mg^{2+} ,

addition of spermine substantially reduced the protein synthesizing activities of both free and bound ribosomes. The observed inhibition is probably due to the excessive aggregation of ribosomes caused by high spermine concentration. There was no striking difference between the protein synthesizing activities of free and bound ribosomes with regard to their stimulation by spermine.

Bound ribosomes were generally more resistant towards the inhibitors used in the present study. This observation is comparable to the results obtained by Schreml and Burka⁴⁰ with reticulocyte ribosomes. At present it is difficult to decide whether the increased resistance of bound ribosomes to the inhibitors is due to the influence of membranes or to some structural difference between free and bound ribosomes.⁴¹ Earlier results from this laboratory, however, have shown that free ribosomes become more resistant to pancreatic ribonuclease after their attachment to membranes. This shows that membrane-attachment may affect the biochemical characteristics of animal ribosomes.

Reattached ribosomes, prepared by the present method, actively participated in both endogenous and poly U-directed phenylalanine incorporation into polypeptide, and acquired some of the characteristics of the authentic bound ribosomes. Since the system permits studies of ribosomes before and after attachment to membranes it may provide a means for further investigation of the effect of membrane-attachment on ribosome function in the animal cell.

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