Intermediate Reactions in the Binding of Aminoacyl-Transfer Ribonucleic Acid to Rat Liver Ribosomes

FORMATION AND PROPERTIES OF AN AMINOACYL-TRANSFER RIBONUCLEIC ACID-TRANSFERASE I COMPLEX

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(Received 5 July 1971)

1. Transferase I of rat liver binds aminoacyl-tRNA to form a relatively stable complex, which is retained on cellulose nitrate filters. This reaction proceeds at both 0°C and 37°C and is inhibited by GTP. The resulting product is stabilized by GTP and Mg^{2+} . 2. Only very low quantities of deacylated tRNA are bound by transferase I. 3. Methods are described for the preparative isolation of the transferase I-aminoacyl-tRNA complex from incubation mixtures by using ion-exchange procedures. 4. The transferase I-aminoacyl-tRNA complex becomes readily bound to ribosomes. The presence of Mg^{2+} is essential for the binding. GTP stimulates this reaction but is not absolutely required. 5. It is concluded that the formation of the transferase I-aminoacyl-tRNA complex may be the primary reaction in the binding of aminoacyl-tRNA to mammalian ribosomes and that, unlike in bacterial systems, GTP is not absolutely required for this step.

Peptide-chain elongation on ribosomes requires three steps: binding of aminoacyl-tRNA to the acceptor site of the ribosome, peptidyl transfer from the donor to the acceptor site, and translocation of peptidyl-tRNA from the acceptor to the donor site (Matthaei *et al.*, 1968).

Binding of aminoacyl-tRNA to the bacterial ribosome is catalysed by an enzyme consisting of a stable (T_s) and unstable (T_u) component (Lucas-Lenard & Lipmann, 1966). The corresponding reaction on the mammalian ribosome requires only one enzyme, the binding enzyme (McKeenan & Hardesty, 1969) or transferase I (Ibuki & Moldave, 1968). The binding of aminoacyl-tRNA to the ribosome in bacterial systems involves several intermediate steps, which have been elucidated recently (Lucas-Lenard *et al.*, 1969). However, there are no experimental results indicating whether the same intermediate reactions occur also on mammalian ribosomes.

Experiments described in this paper were performed to elucidate whether aminoacyl-tRNA becomes bound to the mammalian ribosome by the same mechanism as to the bacterial particle. Evidence was obtained indicating that transferase I of rat liver combines with aminoacyl-tRNA to form an apparently transient complex which may be the initial intermediate in the binding reaction.

Materials and Methods

Biological materials

Wistar rats of both sexes weighing 150–200 g, bred in this Laboratory and kept on a standard diet were

used as the source of rat liver tissue. Animals were starved for 24 h before death.

Chemicals

GTP (sodium salt) was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Poly(U) was a product of Calbiochem, Los Angeles, Calif., U.S.A. Synpor nitrocellulose filters (average pore size $0.60\mu m$) were products of Synthesia, Uhřetíněves, Czechoslovakia.

Radiochemicals

Aminoacyl- $[^{32}P]$ tRNA from *Escherichia coli* was isolated from the commercial preparation of soluble RNA (PB.141) supplied by The Radiochemical Centre, Amersham, Bucks., U.K. This preparation usually contained less than 40% of labelled tRNA, the other components being mainly rRNA and labelled free nucleotides. Aminoacyl- $[^{32}P]$ tRNA was purified from this material by gel filtration on Sephadex G-100 columns. This procedure yielded more than 80% of tRNA present in the original mixture essentially free of radiochemical impurities. The specific radioactivity of such purified preparations was 3000 mCi/mmol of tRNA. $[^3H]$ Phenylalanyl-tRNA from rat liver was prepared as described by Moldave (1963) from $[^3H]$ phenylalanine supplied by The Radiochemical Centre (18 Ci/mmol). Individual preparations contained 5.80–6.70 μ mol of phenylalanine/mmol of tRNA. Aminoacyl- $[^{32}P]$ tRNA was

deacylated by incubation in 1.8M-tris-HCl buffer, pH 8.1, at 37°C for 60 min. The reaction mixture was then dialysed against 0.05M-tris-HCl buffer, pH 7.5, overnight in the cold.

Isolation of transferase I and ribosomes from rat liver

Transferase I was purified as described by Hradec *et al.* (1971). Individual enzyme preparations were 92–108-fold purified with respect to the postmicrosomal supernatant. Ribosomes were isolated and purified free of elongation factors by using the method of Bermek & Matthaei (1970).

Isolation of the transferase I-aminoacyl-tRNA complex

Two methods were developed for a micropreparative separation of this complex from incubation mixtures. If relatively large quantities of both the transfer enzyme and aminoacyl-tRNA were present in the incubation mixture (as in mixtures with [³H]phenylalanyl-tRNA), the mixtures after the incubation were made 250mM with respect to KCl and mixed with a thick suspension of DEAE-cellulose (Whatman DE-50, equilibrated with the washing buffer). The resulting suspension was briefly mixed several times and after 1–2 min centrifuged at low speed at 0°C. The supernatant containing the com-

Fig. 1. Evidence for the binding of aminoacyl-[³²P]tRNA to transferase I

The binding enzyme (34 µg of protein) was incubated with 3.42 pmol of aminoacyl-[³²P]tRNA in 50mM-tris-HCl buffer, pH 7.5, 6mM-MgCl₂ and 50mM-KCl in a total volume of 0.5 ml at 37°C for 15 min. The reaction mixture was then chilled in ice and applied to a column (25mm × 360mm) of Sephadex G-100 equilibrated with a buffer containing 50mM-tris-HCl, pH 7.5, 6mM-MgCl₂ and 50mM-KCl. The column was eluted with the same buffer. The separation was performed at 2°C. The E₂₆₀ of the eluate was continuously registered by a recording absorptiometer. Fractions (2ml) were collected and 0.5ml portions of them were mixed with 10ml of SLS-31 scintillation mixture and assayed for radioactivity. ○, Aminoacyl-[³²P]tRNA; ···, E₂₆₀.

plex was sucked off and quickly frozen in a solid CO_2 -ethanol mixture. It was then stored in solid CO_2 . In the second isolation procedure used in particular for the preparation of complexes with aminoacyl- $[\text{P}^{32}]\text{tRNA}$, the reaction mixture after the incubation was diluted with 5–10 vol. of ice-cold washing buffer (20mM-tris-HCl buffer, pH 7.5, 10mM- MgCl_2 , 20mM-KCl). This solution was then slowly filtered through a double layer of Whatman DE 20 paper prewashed with the washing buffer and the filters were thoroughly washed with the washing buffer afterwards. The complex was then eluted with 0.5–1.0ml of 250mM-KCl in the washing buffer. All these operations were performed at 0°C . The final preparation was quickly frozen in solid CO_2 and kept at -78°C . Both these procedures yielded complexes of transferase I with aminoacyl-tRNA free of un-

bound aminoacyl-tRNA. When stored at the temperature indicated, the complexes were stable for several days. For use in binding experiments, they were quickly thawed immediately before the incubation was started.

Incubation procedures

Mixtures for assay of the enzyme complex formation and its binding to ribosome were prepared and incubated as described by Hradec *et al.* (1971). Mixtures containing ribosomes were diluted to 11ml with ice-cold washing buffer after the incubation and centrifuged at $75000g$ for 60 min at 5°C . Ribosomal pellets were carefully rinsed with ice-cold washing buffer and homogenized in the same medium. The

Fig. 2. Formation of complex A with aminoacyl- $[\text{P}^{32}]\text{tRNA}$ (Δ , $\blacktriangle$) and $[\text{H}^3]\text{phenylalanyl-tRNA}$ ($\circ$, $\bullet$) in the presence ($\blacktriangle$, $\bullet$) and absence (Δ , $\circ$) of GTP

Incubation mixtures contained 50mM-tris-HCl buffer, pH 7.5, 6mM- MgCl_2 , 50mM-KCl, 0.2mM-GTP (where indicated), 0.35 pmol of aminoacyl- $[\text{P}^{32}]\text{tRNA}$ or 262 pmol of $[\text{H}^3]\text{phenylalanyl-tRNA}$ and various quantities of the binding enzyme in a final volume of 0.1 ml. They were incubated at 37°C for 15 min. Incubation mixtures were then filtered through cellulose nitrate filters as described in the Materials and Methods section.

resulting suspensions were then filtered through cellulose nitrate filters.

Assay of radioactivity

Nitrocellulose filters were placed into scintillation vials containing 3 ml of a scintillation mixture [0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene]. Eluates from chromatographic columns were mixed with a SLS-31 scintillation mixture (Tesla, Přemyšlení, Czechoslovakia) in suitable proportions. Radioactivity was assayed with an Ansitron II liquid-scintillation spectrometer (Picker Nuclear, White Plains, N.Y., U.S.A.). Protein and RNA were determined by the method of Warburg & Christian (1942).

Results

Formation of a transferase I-aminoacyl-tRNA complex (complex A)

When transferase I was incubated with GTP and aminoacyl- $^{[32]}\text{P}$ tRNA and the reaction mixture was filtered through nitrocellulose membranes afterwards, a major portion of the total radioactivity was retained on the filter. This was rather unexpected since the ternary complex bacterial T factor-GTP-aminoacyl-tRNA is known to pass through membrane filters (Shorey *et al.*, 1969). Moreover, when GTP was omitted from the reaction mixture an even higher proportion of aminoacyl- $^{[32]}\text{P}$ tRNA remained adsorbed on the filter. These results indicated that aminoacyl-tRNA becomes bound to transferase I even in the absence of GTP. When the reaction mixture was subjected to gel filtration on a Sephadex G-100 column essentially all radioactivity was eluted at a smaller elution volume than that of free aminoacyl-tRNA. Moreover, the peak of the radioactivity coincided exactly with the fractions where the transferase I was also present according to the u.v. absorption (Fig. 1). These results gave a good indication that rat liver transferase I binds aminoacyl-tRNA even in the absence of GTP.

There was a linear proportionality between the amount of transferase I added into incubation mixtures and the quantity of aminoacyl-tRNA retained on membrane filters until the saturation value was reached. GTP in concentrations optimum for the binding of free aminoacyl-tRNA to ribosome had an inhibitory effect on the formation of transferase I-aminoacyl-tRNA complex (complex A). This was true for both $^{[3]}\text{H}$ phenylalanyl-tRNA or aminoacyl- $^{[32]}\text{P}$ tRNA (Fig. 2).

Experiments on the time-course of the complex A formation revealed that aminoacyl-tRNA becomes rapidly bound to transferase I at 37°C with optimum yields after 15 min incubation. However, essentially

the same amount of complex A may be obtained at 0°C although the reaction proceeds more slowly at this temperature (Fig. 3). Again essentially the same results were obtained with $^{[3]}\text{H}$ phenylalanyl-tRNA as with aminoacyl- $^{[32]}\text{P}$ tRNA.

Complex A is highly unstable when incubated at 37°C and becomes completely decomposed after 60 min incubation. At 0°C it is more stable but even at this temperature it becomes gradually split into its original components. However, complex A may be stabilized even at 37°C by the addition of relatively small quantities of GTP. Concentrations of this nucleotide required for maximal stabilization are at least twofold lower than those required for the optimum binding of complex A to ribosome. Complex A is formed in good yields even in the absence of Mg^{2+} . Low concentrations of this ion have an inhibitory effect on the binding of aminoacyl-tRNA to transferase I. However, concentrations of Mg^{2+} in the range 4–8 mM have a significant stimulating effect on the formation of complex A (Fig. 4). No specific requirements of this reaction for univalent cations

Fig. 3. Time-course of complex A formation

Incubation mixtures contained in a final volume of 0.1 ml: 50 mM-tris-HCl buffer, pH 7.5, 6 mM- MgCl_2 , 50 mM-KCl, 3.5 μg of transferase I protein and 0.32 pmol of aminoacyl- $^{[32]}\text{P}$ tRNA. They were incubated at 37°C (○) or 0°C (●) for the time indicated. Reaction mixtures were then filtered through cellulose nitrate filters as described in the Materials and Methods section.

Fig. 4. Effect of Mg^{2+} on the formation of complex A (○) and on the binding of complex A to ribosome (●)

Incubation mixtures for the assay of complex A formation contained in a final volume of 0.1 ml: 50mM-tris-HCl buffer, pH 7.5, 50mM-KCl, 3.5 μ g of transferase I protein and 0.32 pmol of aminoacyl- $[^{32}P]$ tRNA and were incubated at 37°C for 15 min. Incubation mixtures for the assay of the binding of complex A to ribosome contained in a final volume of 0.1 ml: 50mM-tris-HCl buffer, pH 7.5, 50mM-KCl, 2mM-GTP, complex A (5.2 μ g of protein and 0.28 pmol of aminoacyl- $[^{32}P]$ tRNA) and 142 μ g of ribosomes and were incubated at 37°C for 2 min.

could be demonstrated. Essentially the same quantities of the complex were formed in the presence of 0–100mM-KCl.

Transferase I is apparently able to distinguish the aminoacyl-tRNA species from the deacylated species. Small quantities of deacylated $[^{32}P]$ tRNA become bound to transferase I compared with the same aminoacylated preparation. However, a significantly higher proportion of deacylated tRNA (when compared with the results in the complete system) than of aminoacyl-tRNA combines with transferase I in the absence of Mg^{2+} (Table 1). This suggests that Mg^{2+} may play some role in the recognition of aminoacyl-tRNA by the binding enzyme.

Binding of complex A to ribosomes

Binding of free aminoacyl-tRNA to ribosomes in the presence of GTP and transferase I proceeds linearly for about 20min at 37°C after which time a saturation value is apparently reached (Fig. 5). Gel filtration of such incubation mixtures on Sephadex G-100 columns revealed that a minor portion of aminoacyl- $[^{32}P]$ tRNA is eluted together with the

Table 1. Binding of deacylated tRNA by transferase I and by ribosome

Incubation mixtures for the formation of complex A contained in a final volume of 0.1 ml: 50mM-tris-HCl buffer, pH 7.5, 6mM-MgCl₂, 50mM-KCl, 2mM-GTP, 0.35 pmol of aminoacyl- $[^{32}P]$ tRNA or deacylated $[^{32}P]$ tRNA and 4.5 μ g of transferase I protein, and were incubated at 37°C for 15 min. Mixtures for the binding of tRNA to ribosome contained, in addition, 150 μ g of ribosomes and were incubated at 37°C for 30 min. All values are pmol of $[^{32}P]$ tRNA bound to transferase I or ribosome.

System	tRNA	
	aminoacylated	deacylated
Complex A formation		
Complete	0.183	0.042
minus GTP	0.247	0.055
minus GTP+ Mg^{2+}	0.320	0.109
Binding of tRNA to ribosome		
Complete	0.240	0.059
minus GTP	0.194	0.055

Fig. 5. Time-course of the binding of aminoacyl-[³²P]tRNA and complex A to ribosome

Incubation mixtures contained: (a) for the assay of aminoacyl-[³²P]tRNA binding (final volume of 0.1 ml): 50 mM-tris-HCl buffer, pH 7.5, 6 mM-MgCl₂, 50 mM-KCl, 2 mM-GTP, 3.5 μg of transferase I protein and 0.67 pmol of aminoacyl-[³²P]tRNA; (b) for the assay of complex A binding (final volume 0.1 ml): 50 mM-tris-HCl buffer, pH 7.5, 6 mM-MgCl₂, 50 mM-KCl, 2 mM-GTP, complex A (5.2 μg of protein and 0.28 pmol of aminoacyl-[³²P]tRNA) and 142 μg of ribosomes and were incubated at 37°C (○) or 0°C (●).

ribosomes. A significant amount of the radioactivity is eluted as free aminoacyl-tRNA whereas only a small portion is present in the form of complex A (Fig. 6). This suggests that complex A has only a transient existence in such incubation mixtures and that its quantity at a given moment is a result of a dynamic equilibrium between the individual components of the reaction mixture.

Small quantities of deacylated [³²P]tRNA become bound to ribosomes when compared with the aminoacylated species. This amount corresponds roughly to that bound to transferase I (see above) (Table 1). This suggests that transferase I has a role in the discrimination between deacylated and aminoacylated tRNA.

Preformed complex A becomes very rapidly bound to ribosomes in the presence of GTP (Fig. 7). Gel filtration of incubation mixtures to which preformed complex A was added revealed significant differences from those in which free enzyme and aminoacyl-

tRNA were used. Most of the radioactivity was eluted together with ribosomes and only a small proportion of aminoacyl-[³²P]tRNA remained in the form of complex A after the incubation. Moreover, only traces of free aminoacyl-tRNA were present (Fig. 7).

For the binding of complex A to the ribosome GTP is not absolutely required. However, this nucleotide shows a stimulating effect on the binding reaction at an optimum concentration of 0.1 mM. Excessive concentrations of GTP partially inhibit the binding (Fig. 8).

The binding of complex A to ribosomes shows an absolute requirement for Mg²⁺, the optimum concentration being 6–8 mM (Fig. 4).

Discussion

Evidence has been presented by Ertel *et al.* (1968) that the binding enzyme in prokaryotic cells (T

Fig. 6. Composition of fractions obtained by gel filtration of incubation mixtures containing ribosomes, aminoacyl-tRNA and the binding enzyme

The incubation mixture contained in a final volume of 1.0ml: 50mM-tris-HCl buffer, pH 7.5, 6mM-MgCl₂, 50mM-KCl, 2mM-GTP, 58 μ g of transferase I protein, 6.80pmol of aminoacyl-[³²P]tRNA and 1.25mg of ribosomes and was incubated at 37°C for 30min. Gel filtration on Sephadex G-100 was performed as described in the legend for Fig. 1. ○, Aminoacyl-[³²P]tRNA; ···, E₂₆₀.

factor) has a stable and a unstable component. However, only one protein factor (transferase I; Ibuki & Moldave, 1968) is apparently present in mammalian cells. Before a combination of the T factor with aminoacyl-tRNA takes place this factor must react first with GTP (Allende *et al.*, 1967; Shorey *et al.*, 1969). On the other hand, our results give evidence that the combination of mammalian transferase I with aminoacyl-tRNA does not require GTP. Transferase I binds aminoacyl-tRNA readily in the absence of any other cofactors and complex A becomes very rapidly bound to the ribosome even in the absence of GTP. These results seem to indicate that the formation of complex A may be the primary step of the binding reaction in mammalian cells and suggest that the sequence of reactions leading to the codon-directed binding of aminoacyl-tRNA to eukaryotic ribosomes is different from that operating in prokaryotes.

Because of its high specific radioactivity aminoacyl-[³²P]tRNA used in this study showed some advantages when compared with tRNA charged with labelled amino acids most commonly used in such experiments. The only difference between this product and [³H]phenylalanyl-tRNA was a rather rapid loss of label from ribosomes incubated with the former tRNA. This is probably due to the traces of translocase associated with the ribosomes even after their extensive purification; the translocase apparently releases deacylated tRNA from the ribosomes during the translocation step (Lucas-Lenard & Haenni, 1969). Although no phenylalanine polymerization was observed in the absence of added translocase with the ribosome purified by the present procedure, minute quantities of transferase II remaining on the ribosome may nevertheless become significant when aminoacyl-tRNA with unusually high specific radioactivity is used.

Fig. 7. Evidence for the binding of complex A to ribosomes in the absence (a) and presence (b) of GTP

The incubation mixture contained in a final volume of 0.1 ml: 50mM-tris-HCl buffer, pH 7.5, 6mM-MgCl₂, 50mM-KCl, 2mM-GTP (where indicated), complex A (70μg of protein and 6.50pmol of aminoacyl-[³²P]tRNA) and 1.25mg of ribosomes, and was incubated at 37°C for 5 min. Gel filtration on Sephadex G-100 was performed as described in the legend for Fig. 1. ○, Aminoacyl-[³²P]tRNA; ···, E₂₆₀.

No important conclusions can be drawn from the saturation values obtained in the present experiments. The plateaux reached with larger amounts of transferase can apparently only reflect the capacity of the filter for enzyme.

Although GTP does not participate in the formation of complex A it has nevertheless a stabilizing effect on this product. This nucleotide forms a complex with complex A (Hradec, 1972), which is more stable than complex A. This may partly explain the stabilizing effect of GTP, although it is not clear why such low concentrations of this nucleotide (significantly lower than those required for the formation of the ternary complex) are necessary for the stabilization. It has been shown that GTP in the presence of aminoacyl-tRNA is able to protect transferase I completely against inactivation at 37°C in the presence of Mg²⁺ (Ibuki & Moldave, 1968). All these results seem to indicate that complexes containing GTP are the most stable intermediates in the binding reaction.

It is surprising that for the binding of aminoacyl-tRNA to transferase I, Mg²⁺ is not absolutely required although the subsequent binding of complex A to the ribosome is dependent on this ion. It is also not clear why this primary step does not require univalent cations.

Skoultchi *et al.* (1968) have demonstrated that the S₁ and S₃ factors of *Bacillus stearothermophilus* (corresponding to factors T₊ and T_u of *E. coli*) do not bind uncharged tRNA. Similarly in our experiments transferase I does not form a complex with deacylated tRNA. This indicates that the transfer factor is able to discriminate between charged and uncharged species of tRNA, preventing the latter from entering the ribosomal binding sites and interfering with peptide formation. The transfer enzyme is apparently able even to recognize the fine structure of aminoacyl-tRNA, since the T factor does not form the complex with chemically modified tRNA (Chen & Ofengand, 1970).

All the apparent differences between the binding of

Fig. 8. Effect of GTP on the binding of aminoacyl-tRNA from complex A to ribosome

The incubation mixtures contained in a final volume of 0.1 ml: 60 mM-tris-HCl buffer, pH 7.5, 6 mM-MgCl₂, 50 mM-KCl, complex A (5.2 µg of protein and 0.35 pmol of aminoacyl-[³²P]tRNA) and 142 µg of ribosomes. They were incubated at 37°C for 2 min.

aminoacyl-tRNA to bacterial and to rat liver ribosomes are probably characteristic for the binding reaction in mammalian cells and not in eukaryotic cells in general. Thus the binding reaction in yeast cells corresponds closely to that in bacterial systems. Two factors (T₁ and T₃) are required for the binding of

aminoacyl-tRNA to the ribosome (Richter & Klink, 1968). Also a ternary complex of these factors with aminoacyl-tRNA and GTP resembles the complex II of *E. coli* (Richter, 1970).

I thank Dr. Z. Dušek for his help with the isolation of subcellular particles. The excellent technical assistance of Mrs. R. Chalupová and Mrs. Z. Tuhácková is also gratefully acknowledged.

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