rophosphate, and to Dr. Carl Kjeldsberg for photomicrography.

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Studies on the Binding of Acylaminoacyl-tRNA to Rat Liver 60S Ribosomal Subunits and Its Participation in the Peptidyltransferase Reaction†

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ABSTRACT: Peptidyltransferase with rat liver 60S subunits can be measured by the reaction between exogenous acylaminoacyl-tRNA and puromycin to form acylaminoacyl-puromycin in the presence of 33% methanol, 0.3 M KCl, and 4 mM $MgCl_2$. An assay system has been developed that allows examination of the binding of acetylphenylalanyl-tRNA to the ribosomal subunit "P" site, the transpeptidation of the 60S-bound substrate to puromycin, and the requirements for these individual steps. Binding of acetylphenylalanyl-tRNA to 60S subunits is stimulated several-fold by the addition of methanol, but the extent of binding in alcohol is the same in 60 as in 300 mM KCl containing solutions. Formation of acetylphenylalanyl-puromycin from 60S · acetylphenylalanyl-tRNA complex and puromycin stringently requires alcohol and the initial rate of the reaction is markedly greater at 300 mM KCl than at 60 mM KCl concentrations. Thus, alcohol and high concentrations of monovalent cation affect the reaction of an event subsequent to the binding of substrate to the "P" site. Preincubation of 60S subunits with poly(U), which stimulates the overall peptidyltransferase reaction, does not affect the amount of acetylphenylalanyl-tRNA that is bound to the particles; however, it markedly stimulates the initial rate of the transpeptidation reaction between 60S · acetylphenylalanyl-tRNA complex and puromycin. The codon specificity and the failure to affect binding with poly(U) suggest a role for the polynucleotide in the alignment or stabilization of the acylaminoacyl-tRNA on the "P" site rather than an effect on binding to either of the two particle sites or on the peptidyltransferase "active center." The effect of 40S subunits, which inhibit the overall peptidyltransferase reaction, on the binding of substrate could not be clearly interpreted since all three preparations, 60S subunits, 40S subunits, and combinations of 60S plus 40S particles, appear to bind acetylphenylalanyl-tRNA in the presence of methanol. However, the initial rate of peptide bond formation is several times greater with 60S · acetylphenylalanyl-tRNA complex than with 60S plus 40S particles containing bound acetylphenylalanyl-tRNA, and the addition of 40S subunits to preformed 60S · acetylphenylalanyl-tRNA complex during the transpeptidation phase of the reaction in methanol does not affect the rate of peptide bond formation. Thus, 40S subunits seem to inhibit peptidyltransferase by forming less reactive particles in aqueous solutions. Two inhibitors of peptidyltransferase, trichodermin and anisomycin, do not affect binding of substrate to the "P" site, but inhibit a subsequent step in the reaction with 60S-bound substrate.

The formation of peptide bonds in protein synthesis appears to be a property of the ribosomal particle and does not require protein factors or GTP (see review by Lucas-Lenard and Lipmann, 1971). This activity is associated with the large subunit of prokaryote or eukaryote ribosomes (Monro, 1967; Maden *et al.*, 1968; Monro *et al.*, 1969; Vazquez *et al.*, 1969; Falvey and Staehelin, 1970; Ballesta *et al.*, 1971; Nierhaus and Montejo, 1973) and can be assayed by reaction between exogenous donor molecules such as acylaminoacyl-tRNA or acylaminoacyl-oligonucleotide and acceptor molecules such as puromycin, aminoacyl-tRNA, or aminoacyl-oligonucleotide (Monro and Marcker,

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TABLE I: Binding of Acetyl[^{3}H]phenylalanyl-tRNA to Rat Liver 60S Subunits and Its Participation in the Peptidyltransferase Reaction.[a]

First Incubation		Second Incubation			
Components	pmoles of Acetylphenylalanyl-tRNA Sedimented	pmoles of Acetylphenylalanine in Product (−PM)	(+PM)	(Δ)	% of Bound Acetylphenylalanine Converted
60S, AcPhe-tRNA	2.2	0.3	1.3	1.0	45
60S, AcPhe-tRNA, MeOH	17.3	1.8	10.7	8.9	51
AcPhe-tRNA, MeOH	6.6	0.7	0.9	0.2	4

[a] Ribosomal 60S subunits and acetyl[^{3}H]phenylalanyl-tRNA were allowed to react in buffered salts–dithiothreitol solution, in the presence and absence of methanol, at 4° for 10 min (first incubation), as described in Binding of Acetylaminoacyl-tRNA to 60S Subunits. The 60S subunits were sedimented from the reaction mixtures, and analyzed for bound acetylphenylalanyl-tRNA; duplicate sets were resuspended and assayed for peptidyltransferase activity in the presence of methanol and 0.3 M KCl, with (+PM) and without (−PM) puromycin for 30 min (second incubation), as described above in Peptidyltransferase Assay.

1967; Monro *et al.*, 1969; Pestka, 1970; Monro, 1971; Lessard and Pestka, 1972; Hussain and Ofengand, 1972; Nierhaus and Montejo, 1973). Peptidyltransferase, with isolated 50S or 60S subunits, requires relatively high concentrations of alcohol and monovalent cations (Monro, 1967; Maden *et al.*, 1968; Monro *et al.*, 1969; Vazquez *et al.*, 1969; Falvey and Staehelin, 1970; Thompson and Moldave, 1974). Recent studies in this laboratory (Thompson and Moldave, 1974) indicated that rat liver 60S subunits catalyzed the formation of acetylaminoacyl-puromycin from acetylaminoacyl-tRNA and puromycin; this reaction was inhibited by preincubation of the 60S particles with 40S subunits and it was stimulated by preincubation with polynucleotides having the appropriate codon composition. The present study describes the effects of various components that influence peptidyltransferase on the binding of acetylaminoacyl-tRNA to the 60S subunits and on the subsequent reaction between the particle-bound substrate and puromycin.

Materials and Methods

The preparation of purified ribosomes and ribosomal subunits from rat liver (Skogerson and Moldave, 1967, 1968; Martin and Wool, 1968; Gasior and Moldave, 1972; Thompson and Moldave, 1974) and of ^{3}H-labeled acetylphenylalanyl-tRNA have been described (Haenni and Chapeville, 1966; Siler and Moldave, 1969; Thompson and Moldave, 1974). The specific activity of the tRNA-bound [^{3}H]phenylalanine was 5300 cpm/pmol of amino acid and preparations varied between 1500 and 3000 cpm/μg of acylaminoacyl-tRNA.

Binding of Acetylaminoacyl-tRNA to 60S Subunits. Approximately 385 pmol of 60S subunits was maintained at 4° in buffered salts–dithiothreitol solution (40 mM Tris-HCl (pH 7.5)–300 mM KCl–4 mM $MgCl_2$–1.3 mM dithiothreitol), 33% methanol, and an amount of acetylphenylalanyl-tRNA containing 60 pmol of [^{3}H]phenylalanine. In some cases, methanol or acetylphenylalanyl-tRNA was omitted from this phase of the experiment; in some, the KCl concentration was 60 mM. The total volume was 0.45 ml. After 10 min, the reaction mixture was diluted with 3 ml of the buffered salts–dithiothreitol solution and centrifuged at 100,000*g* for 4 hr. The sedimented pellet was resuspended in water and counted with a scintillation counter to determine the amount of 60S-bound acetyl[^{3}H]phenylalanyl-tRNA. Pellets obtained from duplicate reaction mixtures were assayed for peptidyltransferase activity as described below.

Peptidyltransferase Assay. Approximately 385 pmol of 60S subunits obtained after centrifugation and resuspended in buffered salts–dithiothreitol, or untreated 60S controls, were incubated at 20° with or without 33% methanol, in the presence and absence of 0.8 mM puromycin. Final assay conditions and components were as described previously (Thompson and Moldave, 1974). Those samples that did not contain labeled acetylphenylalanyl-tRNA in the binding phase of the reaction at 4° received an amount of substrate equivalent to that which sedimented with the 60S subunits in the binding assay; other components used in the 4° phase of the reaction were also added to control (nonpreincubated) 60S preparations. The total volume was 0.45 ml and the final concentration of the buffered salts–dithiothreitol was as noted in the binding phase of the reaction. At the end of a 10- or 30-min incubation period, the samples were analyzed for labeled acetylphenylalanyl-puromycin (Leder and Bursztyn, 1966; Maden and Monro, 1968). The results are expressed as picomoles of ^{3}H-labeled amino acid extracted with ethyl acetate; in most cases the amount of radioactivity extracted with ethyl acetate in incubations without puromycin has been subtracted.

Results

When 60S subunits and acetyl[^{3}H]phenylalanyl-tRNA were allowed to react for 10 min at 4°, approximately 4% of the exogenous acetylphenylalanyl-tRNA was recovered with the 60S subunits obtained by centrifugation (Table I, line 1). Incubation of the resuspended 60S · acetylphenylalanyl-tRNA complex at 20° with methanol and puromycin indicated that about 45% of the particle-bound substrate was converted to the puromycin derivative in a 30-min incubation. Similar experiments with methanol in the first phase of the experiment (line 2) revealed that about 29% of the exogenous acetylphenylalanyl-tRNA was bound to 60S subunits; as with the 60S · acetylphenylalanyl-tRNA complex prepared in the absence of methanol, about 50% of the particle-bound substrate then reacted with puromycin to yield acetylphenylalanyl-puromycin. In the experiments described below, with a number of labeled acetylphenylalanyl-tRNA preparations, 3–5 times more radioactivity was sedimented from the methanolic solutions when 60S subunits

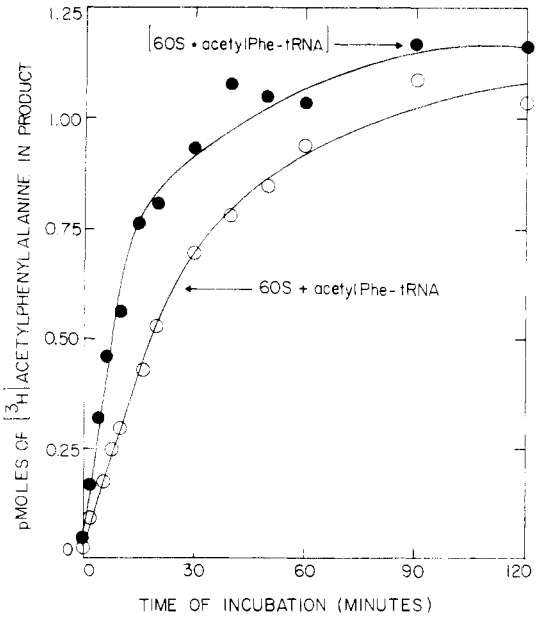

FIGURE 1: Time dependent formation of acetylphenylalanyl-puromycin from 60S-bound acetyl[^{3}H]phenylalanyl-tRNA (●) and from free acetyl[^{3}H]phenylalanyl-tRNA (○). The preincubation of 60S subunits with methanol, in the presence and absence of acetylphenylalanyl-tRNA, was carried out as described under Materials and Methods but contained three times the amounts of 60S subunits (1190 pmol) and of acetyl[^{3}H]phenylalanyl-tRNA (191 pmol). After sedimentation and resuspension, a duplicate of the incubation with labeled substrate was counted, and an equivalent amount of acetyl[^{3}H]phenylalanyl-tRNA was added to the 60S subunits that were incubated without labeled substrate. Both suspensions were then incubated at 20° for 120 min, with methanol and puromycin as described under Peptidyltransferase Assay. At various times, aliquots were withdrawn and analyzed for ethyl acetate extractable material. The results are expressed as picomoles of isotopically labeled phenylalanine recovered in the ethyl acetate fraction of individual aliquots, after subtraction of the values obtained from incubations without puromycin.

were present, as compared to incubations in which 60S subunits were omitted (line 3).

The kinetics of acetylphenylalanyl-puromycin formation, from free and from 60S subunit-bound acetylphenylalanyl-tRNA, are shown in Figure 1. The initial rate of the peptidyltransferase reaction was 2 to 2.5 times greater with 60S · acetyl[^{3}H]phenylalanyl-tRNA complex (closed circles) than with 60S subunits incubated with equivalent amounts of free acetyl[^{3}H]phenylalanyl-tRNA (open circles). Under these conditions, both the 60S subunit bound and the free substrate were almost completely utilized during the 120-min incubation period.

The effect of alcohol on binding and on the initial kinetics of the peptidyltransferase reaction was investigated. In this and in all subsequent experiments, the reaction with puromycin was carried out for 10 min in order to study initial rates (Thompson and Moldave, 1974). As shown in Table II, when the binding phase of the reaction was carried out in the absence of methanol (line 1), about 5% of the acetylphenylalanyl-tRNA was bound to the 60S subunits sedimented by centrifugation. Incubation of this complex, in the peptidyltransferase assay with methanol and puromycin, revealed that approximately 20% of the bound acetylphenylalanyl-tRNA was converted to acetylphenylalanyl-puromycin. When 60S subunits were maintained at 4° without acetylphenylalanyl-tRNA then sedimented and incubated in the peptidyltransferase assay with 3 pmol of substrate (line 2), the conversion of about 20% of the free acetylphenylalanyl-tRNA to the puromycin derivative was catalyzed. When methanol was present in both the binding and the peptidyltransferase steps of the reaction (line 3), the initial rate of the reaction with preformed 60S · acetylphenylalanyl-tRNA complex was several (2.5 to 4) times greater than with 60S particles incubated in the transpeptidation reaction with 13 pmol of free acetylphenylalanyl-tRNA (lines 4 and 5). Although in this experiment the formation of acetylphenylalanyl-puromycin from free radioactive acetylphenylalanyl-tRNA was slightly greater with untreated 60S particles (line 5) as compared to 60S subunits preincubated with methanol (line 4), this is not a consistent finding; in other experiments, methanol-treated particles were slightly more active than untreated subunits. When methanol was omitted from the second (peptidyltransferase) phase of the reaction only, acetylphenylalanyl-puromycin formation was not carried out by 60S subunits with

TABLE II: The Effect of Methanol on the Binding of Acetyl[^{3}H]phenylalanyl-tRNA to Rat Liver 60S Subunits and on Peptidyltransferase.[a]

First Incubation		Second Incubation	
Components	pmoles of AcPhe-tRNA Sedimented	Additions	pmoles of AcPhe-puromycin Formed
60S, AcPhe-tRNA	3.2	MeOH, puromycin	0.6
60S		MeOH, puromycin, AcPhe-tRNA[b]	0.6
60S, AcPhe-tRNA, MeOH	13.8	MeOH, puromycin	4.0
60S, MeOH		MeOH, puromycin, AcPhe-tRNA[c]	1.0
60S		MeOH, puromycin, AcPhe-tRNA[c]	1.6
60S, AcPhe-tRNA, MeOH	18.5	Puromycin	0.1
60S, MeOH		Puromycin, AcPhe-tRNA[d]	0

[a] Ribosomal 60S subunits were maintained at 4° for 10 min in buffered salts–dithiothreitol solution, with and without labeled acetylphenylalanyl-tRNA or methanol (first incubation). The 60S subunits were sedimented and one set was analyzed for bound acetylphenylalanyl-tRNA. The other samples were resuspended and incubated with or without puromycin or methanol for 10 min (second incubation); samples that had not received acetylphenylalanyl-tRNA in the binding step ,received 3,[b] 13,[c] or 18[d] pmol of acetyl[^{3}H]phenylalanyl-tRNA per incubation.

TABLE III: The Effect of Monovalent Cation on the Binding of Acetyl[^{3}H]phenylalanyl-tRNA to Rat Liver 60S Subunits and on Peptidyltransferase.[a]

First Incubation		Second Incubation	
KCl Concn (mM)	pmoles of AcPhe-tRNA Sedimented	KCl Concn (mM)	pmoles of AcPhe-puromycin Formed
60	14.9	60	1.0
60	14.9	300	4.5
300	15.8	60	1.2
300	15.8	300	4.1

[a] Ribosomal 60S subunits were allowed to react with acetyl-[^{3}H]phenylalanyl-tRNA and methanol at 4° for 10 min, in buffered salts–dithiothreitol solutions containing 60 or 300 mM KCl as noted (first incubation). The 60S subunits were sedimented and one set from the low salt and one set from the high salt mixtures were analyzed for bound acetylphenylalanyl-tRNA. The other samples were resuspended in buffered salts–dithiothreitol solutions containing 60 or 300 mM KCl and incubated with methanol, in the presence and absence of puromycin (second incubation).

prebound (line 6) or with 18 pmol of free (line 7) acetylphenylalanyl-tRNA.

Similar studies on the effect of monovalent cation on binding and on transpeptidation are summarized in Table III. Incubation of 60S subunits and radioactive acetylphenylalanyl-tRNA, in 60 or 300 mM KCl containing solutions, did not affect the total amount of substrate recovered with the particles sedimented from the reaction mixture. However, when the peptidyltransferase phase of the reaction was carried out with 300 mM KCl (lines 2 and 4) the rate of formation of acetylphenylalanyl-puromycin was three to four times greater than that obtained with 60 mM KCl.

Previous studies in this laboratory (Thompson and Moldave, 1974) indicated that poly(U) stimulated the 60S-catalyzed reaction between puromycin and acetylphenylalanyl-tRNA, especially if the polynucleotide was allowed to react with 60S subunits prior to the peptidyltransferase assay. In order to determine whether this observation could be explained simply on the basis of an effect on the binding of acetylphenylalanyl-tRNA to the particle, acetylphenylalanyl-tRNA was allowed to react with 60S subunits that had been incubated briefly with and without poly(uridylic acid) (Table IV). When nonpreincubated 60S subunits (line 1) or 37°-preincubated 60S particles (line 2) were maintained with acetyl[^{3}H]phenylalanyl-tRNA for 10 min at 4°, 30–35% of the exogenous acetylphenylalanyl-tRNA was recovered with the 60S subunits obtained by centrifugation. The addition of poly(U) to the preformed 60S · acetylphenylalanyl-tRNA complex (line 3) stimulated the reaction between the 60S-bound acetylphenylalanyl-tRNA and puromycin about twofold. Preincubation of the particle at 37° with poly(U) (line 4) appeared to inhibit slightly the amount of 60S-bound acetylphenylalanyl-tRNA recovered; in several such experiments, poly(U) either inhibited binding to a small extent or had no effect. Transpeptidation with 60S · acetylphenylalanyl-tRNA complex prepared with particles preincubated with poly(U) was also markedly greater (line 4) than that observed with complex formed with 60S subunits which were preincubated without polyuridylate (line 2).

The effect of poly(U) on the binding of acetylphenylalanyl-tRNA to 60S subunits without methanol, although not shown here, was also investigated. Approximately 2–3 pmol of acetylphenylalanyl-tRNA were sedimented from buffered salts solution when 60S subunits were present, as also shown above in Tables I and II; when poly(U) was added to these incubations, the amount of radioactivity associated with these sedimented 60S particles was also routinely less (between 1 and 2 pmol) than when poly(U) was omitted.

The binding of acetylphenylalanyl-tRNA to 40S and 60S subunits, individually and together, and the formation of acetylphenylalanyl-puromycin with these particles, is summarized in Table V. The control incubation with 60S subunits alone (line 1) indicated that about 21% of the added radioactive substrate was associated with the subunits sedi-

TABLE IV: The Effect of Poly(uridylic acid) on the Binding of Acetyl[^{3}H]phenylalanyl-tRNA to Rat Liver 60S Subunits and on Peptidyltransferase.[a]

	Second Incubation		Third Incubation	
First Incubation Components	Additions	pmoles of AcPhe-tRNA Sedimented	Additions	pmoles of AcPhe-puromycin Formed
None	60S, AcPhe-tRNA, MeOH	22.5	MeOH, puromycin	3.9
60S	AcPhe-tRNA, MeOH	22.0	MeOH, puromycin	2.6
60S	AcPhe-tRNA, MeOH	22.0	MeOH, puromycin, poly(U)	5.6
60S, poly(U)	AcPhe-tRNA, MeOH	16.8	MeOH, puromycin	6.1

[a] Ribosomal 60S subunits were incubated at 37° for 5 min in a solution containing 50 mM Tris-HCl (pH 7.3), 50 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, and 0.35 M sucrose, with and without poly(uridylic acid) (first incubation). Control and preincubated 60S subunits were then allowed to react with acetyl[^{3}H]phenylalanyl-tRNA and methanol at 4° for 10 min (second incubation). The 60S subunits were sedimented and one set of samples was analyzed for bound acetylphenylalanyl-tRNA. The other samples were resuspended and incubated with methanol, in the presence and absence of puromycin (third incubation); one set of the samples preincubated without polynucleotide template received poly(U).

TABLE V: The Effect of 40S Subunits on the Binding of Acetyl[^{3}H]phenylalanyl-tRNA to Ribosomal Particles and on Peptidyltransferase.

First Incubation Components	Second Incubation: Additions	Second Incubation: pmoles of AcPhe-tRNA Sedimented	Third Incubation: Additions	Third Incubation: pmoles of AcPhe-puromycin Formed
60S	AcPhe-tRNA + MeOH	12.8	MeOH, puromycin	2.8
60S	AcPhe-tRNA + MeOH	12.8	MeOH, puromycin 40S	2.5
40S	AcPhe-tRNA + MeOH	9.4	MeOH, puromycin	0.1
60S + 40S	AcPhe-tRNA + MeOH	13.6	MeOH, puromycin	0.8

[a] Ribosomal 60S and 40S subunits were incubated individually or together, at 37° for 5 min, in a solution containing 50 mM Tris-HCl (pH 7.3), 50 mM KCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, and 0.35 M sucrose (first incubation). The preincubated particles were then allowed to react with acetyl[^{3}H]phenylalanyl-tRNA and methanol at 4° for 10 min (second incubation). The subunits were sedimented and one set of samples was analyzed for bound acetylphenylalanyl-tRNA. The other samples were resuspended and incubated with methanol, in the presence and absence of puromycin (third incubation); one set of the preincubated 60S subunits received 40S subunits.

mented, and that 20–25% of the bound material reacted with puromycin in 10 min. When 40S subunits were added to the isolated 60S · acetylphenylalanyl-tRNA complex, for the peptidyltransferase step, the subsequent reaction with puromycin was not significantly affected (line 2); this observation was consistent with the results presented previously (Thompson and Moldave, 1974) which indicated that inhibition of peptidyltransferase was obtained when 40S subunits were preincubated with 60S subunits, prior to the peptidyltransferase step with puromycin. Incubation of radioactive acetylphenylalanyl-tRNA with 40S subunits also led to the binding of significant amounts of radioactivity to this particle (line 3); however, the 40S-bound material did not participate in the peptidyltransferase reaction with puromycin. When 60S and 40S subunits were incubated briefly at 37° in the absence of methanol (line 4), then maintained with labeled substrate and methanol, about 23% of the added acetylphenylalanyl-tRNA was recovered with the pellets sedimented from the methanolic solution. Although the amount of acetylphenylalanyl-tRNA in the 60S plus 40S pellet was quantitatively similar to that obtained in the experiments with 60S subunits alone (lines 1 and 2), the amount of acetylphenylalanyl-puromycin formed was markedly less, about ⅓ of that formed with 60S subunits.

The addition of 10^{-4} M trichodermin or anisomycin to the peptidyltransferase assay system inhibited the reaction 78 and 86%, respectively. The effects of these antibiotics on the binding and transpeptidation reactions are shown in Table VI. The binding of acetylphenylalanyl-tRNA to 60S subunits was not affected by the presence of 10^{-4} M trichodermin or anisomycin. In the trichodermin set of experiments (lines 1–3), approximately 35% of the 74 pmol of acetylphenylalanyl-tRNA added was associated with the 60S subunits sedimented from the reaction; in the set of incubations dealing with the effect of anisomycin (lines 4–6), approximately 20% of the 64 pmol of acetylphenylalanyl-tRNA added was sedimented with the 60S subunits. When the incubations were carried out in the complete absence of trichodermin (line 1) or anisomycin (line 4), about 33% of the bound acetylphenylalanyl-tRNA was converted to the puromycin product. However, the preformed 60S · acetylphenylalanyl-tRNA complex prepared in the presence of trichodermin (line 2) or anisomycin (line 5), converted 20–25% of the bound substrate to the puromycin derivative suggesting that some antibiotic remained with the 60S particle through the centrifugation procedure (Barbacid and Vazquez, 1974). Control, preformed 60S complexes prepared in the absence of antibiotics, were almost completely inhibited when the peptidyltransferase assay with puromycin was carried out in the presence of trichodermin (line 3) or anisomycin (line 6).

Discussion

The formation of peptide bonds during polypeptide chain elongation occurs when peptidyl-tRNA is present on the P site of 80S ribosomes and the A site is occupied by aminoacyl-tRNA. An activity on the large subunit of the ribosomes, peptidyltransferase, catalyzes the transpeptidation reaction between the peptide moiety of the peptidyl-tRNA and the amino group of aminoacyl-tRNA, resulting in the formation of a new peptidyl-tRNA at the A site. The P site is filled by translocation of the newly formed peptidyl-tRNA from the A site, and requires elongation factor EF-2 and GTP; the A site is filled by a new aminoacyl-tRNA, in a codon-dependent reaction which requires elongation factor EF-1 and GTP. In the experiments described in this communication, a number of intermediary steps appear to be involved (Pestka *et al.*, 1970) which differ in some respects from those that occur in protein synthesis. The peptidyl-tRNA analog, acylaminoacyl-tRNA, reacts directly with the P site of the 60S subunit and the aminoacyl-tRNA analog, puromycin, reacts with the A site; peptidyltransferase then catalyzes the formation of acylaminoacyl-puromycin, and some preliminary evidence in this laboratory suggests that most of the product is released from the ribosomal subunit.

The binding of acetylphenylalanyl-tRNA to the 60S particle is markedly stimulated by the presence of alcohol, but occurs to the same extent in solutions containing relatively low (60 mM) or high (300 mM) concentrations of monovalent cation. The acylaminoacyl-tRNA that is bound both in the presence and absence of alcohol participates very effec-

TABLE VI: The Effects of Trichodermin and Anisomycin on the Binding of Acetyl[^{3}H]phenylalanyl-tRNA to Rat Liver 60S Subunits and on its Participation in the Peptidyltransferase Reaction.[a]

First Incubation		Second Incubation		
Components	pmoles of AcPhe-tRNA Sedimented	Additions	pmoles of AcPhe-puromycin Formed	Per Cent Activity
60S, AcPhe-tRNA, MeOH	25.4	MeOH, puromycin	8.3	100
60S, AcPhe-tRNA, MeOH, trichodermin	24.6	MeOH, puromycin	5.2	63
60S, AcPhe-tRNA, MeOH		MeOH, puromycin, trichodermin	1.3	15
60S, AcPhe-tRNA, MeOH	11.0	MeOH, puromycin	4.0	100
60S, AcPhe-tRNA, MeOH, anisomycin	12.4	MeOH, puromycin	3.2	81
60S, AcPhe-tRNA, MeOH		MeOH, puromycin, anisomycin	0.2	5

[a] Ribosomal 60S subunits were allowed to react with acetyl[^{3}H]phenylalanyl-tRNA and methanol at 4° for 10 min, in buffered salts–dithiothreitol solution, in the presence and absence of trichodermin or anisomycin (first incubation). The 60S subunits were sedimented and one set with and without trichodermin and one set with and without anisomycin were analyzed for bound acetylphenylalanyl-tRNA. The other samples were resuspended and incubated with methanol, in the presence and absence of puromycin (second incubation); one set of samples preincubated without antibiotic received trichodermin and another received anisomycin. The results are expressed as pmoles of radioactive phenylalanine extracted from complete incubations, after subtraction of the values obtained from incubations without puromycin, and as per cent of peptidyltransferase activity as compared to reactions without trichodermin or anisomycin, respectively.

tively in the peptidyltransferase reaction. About 50% of the 60S · acylaminoacyl-tRNA complexes formed in the presence or absence of alcohol reacts with puromycin when incubated in the peptidyltransferase assay for 30 min; this suggests that although the amount of substrate sedimented with the particles in the presence of alcohol is several times greater than that obtained in the absence of alcohol, the acetylphenylalanyl-tRNA bound with alcohol is also in the appropriate position on the P site to react with puromycin. Studies with acylaminoacyl-oligonucleotide fragments indicate that binding of acetylleucyl-oligonucleotide to the P site of 50S subunits does not occur in the absence of alcohol (Celma *et al.*, 1970), and that binding of aminoacyl-oligonucleotide to the A site of 70S ribosomes is markedly stimulated by alcohol (Pestka *et al.*, 1970; Lessard and Pestka, 1972; Harris and Pestka, 1973). In contrast to the results on the binding phase of the reaction, alcohol and a high concentration of KCl (300 mM) are essential for transpeptidation when the particle-bound complexes are incubated with puromycin.

The observation that binding is effective when the levels of KCl are relatively low suggests that higher concentrations of monovalent cation may be required for the interaction of substrate with the A site, as indicated by studies on the binding of some acylaminoacyl-oligonucleotide fragments to 70S ribosomes (Pestka *et al.*, 1970; Ringer and Chladek, 1974), or for the peptidyltransferase reaction between 80S-bound peptidyl-tRNA and aminoacyl-tRNA (Skogerson and Moldave, 1968); indeed, experiments in this laboratory indicate that although the formation of peptidyl-puromycin is stimulated by increasing amounts of monovalent cation, the factor-dependent binding of aminoacyl-tRNA is inhibited at concentrations above 80 mM, and the high Mg^{2+}-dependent, factor-independent binding is not affected. Whether in these experiments with 60S subunits, methanol, and puromycin, KCl is required for the binding of the A-site substrates (including aminoacyl-tRNA and aminoacyl-oligonucleotide), or for transpeptidation, or both, is currently under investigation.

Previous studies (Thompson and Moldave, 1974) indicate that when 60S subunits are preincubated with poly(uridylic acid), the subsequent reaction between acetylphenylalanyl-tRNA and puromycin is stimulated several-fold. The stimulation appears to be codon specific since other polynucleotides decrease the initial rate of the reaction with acetylphenylalanyl-tRNA and poly(U) decreases the initial rate of the reaction with other acylaminoacyl-tRNAs. These observations were interpreted to mean that poly(U) enhanced the amount of acetylphenylalanyl-tRNA bound to the P site of the 60S subunit, or that it affected the stabilization or proper alignment of the bound substrate. The possibility that poly(U) affected the ribosomal peptidyltransferase "active center" was not seriously considered because poly(U) has no effect or inhibits transpeptidation with other acylaminoacyl-tRNAs; the possibility that the polynucleotide affects the interaction of substrate at the A site is inconsistent with its specificity for the anticodon composition of the P site substrate, and the use of puromycin which would not be expected to recognize polynucleotide templates. The data presented here indicate that the poly(U) effect cannot be explained by a direct stimulation of the amount of acetylphenylalanyl-tRNA that is bound to the P site of 60S subunits. Additional studies indicate that the apparent K_m for the acetylphenylalanyl-tRNA reaction with poly(U) -treated 60S subunits is not significantly different from that obtained with control subunits; poly(U) increases both the V_{max} and the extent of the reaction. These data and the failure of poly(U) to increase the binding of acetyl-

phenylalanyl-tRNA is thus more consistent with the interpretation that polynucleotide templates enhance the stabilization or proper alignment of the acylaminoacyl-tRNA that is bound to the P site.

Previous studies (Thompson and Moldave, 1974) also indicated that when 60S subunits were preincubated with 40S particles, the subsequent reaction between acetylphenylalanyl-tRNA and puromycin in methanolic solutions was markedly inhibited. It was suggested that the presence of 40S subunits could interfere with the binding of the tRNA derivative to 60S particles, or that an 80S ribosome is formed which either binds acylaminoacyl-tRNA to the puromycin-unreactive A site only or is inactive in the peptidyltransferase assay with methanol. Whether 40S subunits affect the binding of acetylphenylalanyl-tRNA cannot be clearly interpreted from the centrifugation data presented here; 60S and 40S subunits individually bind acetylphenylalanyl-tRNA and when 60S plus 40S subunits are combined, the amount of acylaminoacyl-tRNA bound is not significantly different from that obtained with 60S subunits alone. Complexes formed with acetylphenylalanyl-tRNA, poly(U), and 80S ribosomes (Siler and Moldave, 1969) or 60S plus 40S subunits (Schroer and Moldave, 1973) do not react with puromycin in aqueous solutions unless EF-2 and GTP are present, suggesting that the acylaminoacyl-tRNA is bound initially to the A site of 80S ribosomes and is translocated to the reactive P site with EF-2 and GTP. However, the reaction between puromycin and these 80S · acetylphenylalanyl-tRNA complexes containing the bound substrate on the P site is markedly inhibited in 33% methanol, in contrast to experiments with 60S · acetylphenylalanyl-tRNA; thus, whereas 60S subunits require relatively high concentrations of alcohol for peptidyltransferase, 80S ribosomes are inhibited. More recent experiments (Edens *et al.*, 1974) indicate that 40S subunits cause only a slight decrease in the rate of transpeptidation between acetylphenylalanyl-oligonucleotide and 60S subunits in the methanolic peptidyltransferase assay, whereas the reaction with acetylphenylalanyl-tRNA is markedly inhibited. This finding suggests that 40S subunits may interfere with the proper alignment of the bound acylaminoacyl-tRNA, as consequence of its size, in such a manner as to affect its reactivity in peptidyltransferase. The present data also indicate that 40S subunits, when added to preformed 60S · acetylphenylalanyl-tRNA complexes, do not affect the interaction of puromycin with the A site of the ribosome or the peptidyltransferase "active center;" they further suggest that the 40S subunits do not interact with 60S particles or with 60S · acetylphenylalanyl-tRNA complex in methanolic solutions.

The antibiotics trichodermin and anisomycin have been reported to inhibit peptidyltransferase and peptide chain termination (Carrasco *et al.*, 1973; Tate and Caskey, 1973; Wei *et al.*, 1974). Tate and Caskey (1973) found that trichodermin inhibited the formation of fMet-puromycin and the release factor-dependent dissociation of formylmethionine from ribosome-bound formylmethionyl-tRNA. Carrasco *et al.*, (1973) reported that trichodermin (and anisomycin) inhibited partly the binding of acylaminoacyl- and aminoacyl-oligonucleotide fragments to 80S ribosomes; the Mg^{2+}-dependent, factor-independent binding of acetylphenylalanyl-tRNA and the factor-dependent binding of phenylalanyl-tRNA was not affected by the antibiotics. The present data are consistent with a site of action for these antibiotics other than the P-site interaction. The possibility exists that in contrast to the tRNA derivatives, binding of acylaminoacyl-oligonucleotide fragments, which appear to bind weakly to particles, or their orientation on the P site, is affected by the antibiotics. Experiments in progress with a number of different P site and A site substrates such as acylaminoacyl-oligonucleotides, aminoacyl-oligonucleotides, and aminoacyl-tRNAs are designed to examine in greater detail the nature of the interaction with the two ribosomal sites and the effect of polynucleotides and of 40S subunits on their interactions.

Acknowledgments

The technical assistance of Mrs. Eva Mack, Mr. Arthur Coquelin, and Mr. Robert Sawchuk is gratefully acknowledged. The trichodermin and anisomycin were gifts from Dr. C. McLaughlin.

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Evidence for Multiple Forms and Partial Resolution of Rabbit Reticulocyte α- and β-Globin Messenger RNA by Gel Isoelectric Focusing†

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ABSTRACT: Isoelectric focusing in polyacrylamide gels has been used to fractionate and characterize RNAs from rabbit reticulocytes with major emphasis on globin mRNA. Reticulocyte 18S and 28S RNAs banded essentially as single components, well separated from each other and from the multiple forms of tRNA. By contrast, mRNA was resolved into a number of major and minor components. These bands were shown to contain intact globin mRNA by translation in a messenger-dependent cell-free protein synthesizing system. One major band was enriched slightly in α-globin mRNA and a second major band was enriched considerably in β-globin mRNA. Reticulocyte supernatant mRNA, containing predominantly α-globin messenger, demonstrated only one major component which banded at the same position as the α-enriched band from total mRNA. Little of this material behaved as β mRNA either by its focusing profile or by its translation products. Globin mRNA fractions with high and low 3′ poly(A) contents also demonstrated differences in focusing distribution profiles. Although the basis for separating RNA by this technique has not been established, our results suggest that isoelectric focusing may offer a new approach to fractionation and characterization of specific mRNA species.

In the past few years, there has been considerable interest in the isolation and purification of eukaryotic mRNAs. Messenger fractions active in the synthesis of specific proteins have been prepared by a variety of methods (Moldave and Grossman, 1974). Such preparations have been valuable for direct translation studies and for synthesis of labeled complementary DNA (Ross *et al.*, 1972a; Verma *et al.*, 1972; Kacian *et al.*, 1972) to examine gene expression at both transcriptional and post-transcriptional levels (Ross *et al.*, 1972b; Housman *et al.*, 1973; Terada *et al.*, 1972; Macnaughton *et al.*, 1974).

In efforts to further purify and characterize specific mRNAs, we have explored the potential usefulness of gel isoelectric focusing. We chose the rabbit reticulocyte system as a model, since it has been well characterized and can serve as an excellent source for both mRNA and other cell-free translation components. The reticulocyte is particularly attractive for studying the properties of mRNA since (1) the bulk of its mRNA codes for α- and β-globin chains, (2) the synthesis of α- and β-globin polypeptides can be quantitated readily by well-established procedures, and (3) the focusing behavior of these two distinct but closely related mRNA species can be followed simultaneously. Our results suggest that gel isoelectric focusing may, indeed, be useful for separating discrete mRNAs from each other as well as from other RNA components. They also indicate that α- and β-globin mRNA may exist in multiple molecular forms. Preliminary accounts of these studies have been reported previously (Shafritz *et al.*, 1973b; Shafritz and Drysdale, 1974).

Experimental Procedure

Materials

All chemicals were of analytical or reagent grade and solutions were freshly prepared. Enzyme grade ammonium sulfate, ribonuclease-free sucrose, unlabeled L-amino acids, and [^{3}H]poly(U),[1] K^+ salt (specific activity 7.76 Ci/mol of phosphorus), were purchased from Schwarz/Mann, Oran-

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[1] Abbreviations used are: Temed, N,N,N′,N′-tetramethylenediamine; poly(U), poly(uridylic acid); P-enolpyruvate, phosphoenolpyruvate.