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Activity of the 2′ and 3′ Isomers of Aminoacyl Transfer Ribonucleic Acid in the in Vitro Peptide Elongation on *Escherichia coli* Ribosomes†

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ABSTRACT: Properties of Phe-tRNA^{Lys}-CpCp3′dA and Lys-tRNA^{Lys}-CpCp2′dA from *Escherichia coli* were studied in partial reactions of protein biosynthesis using the *E. coli* in vitro translation system. These aminoacyl-tRNAs carry the aminoacyl residue on either the 2′- or 3′-hydroxyl group of the terminal adenosine. The migration of the aminoacyl residue is restricted by the absence of the vicinal hydroxyl group. Both aminoacyl-tRNA^{Lys} analogues interact with the elongation factor Tu. EF-Tu stimulates the binding of both isomers to 70S ribosomes. However, Phe-tRNA^{Lys}-CpCp3′dA exhibited a higher level of EF-Tu-dependent binding to the ribosomal A site as compared to Lys-tRNA^{Lys}-CpCp2′dA. The nonenzymatic binding was considerably more efficient in the case of the latter analogue, in which the aminoacyl residue is attached to the 3′ position. Lys-tRNA^{Lys}-CpCp2′dA is active as a peptide acceptor and Ac_2Lys-tRNA^{Lys}-CpCp2′dA as a peptide donor. The corresponding 2′ isomers derived from tRNA^{Lys}-CpCp3′dA do not form dipeptides under these conditions. The peptidyl-tRNA^{Lys}-CpCp2′dA binds directly to the ribosomal P site, as indicated by the absence of stimulation of the puromycin reaction by the elongation factor G. The aminoacyl- or peptidyl-tRNAs derived from tRNA^{Lys}-CpCp2′dA are active in all partial reactions of the protein elongation cycle investigated; however, Lys-tRNA^{Lys}-CpCp2′dA is still not able to participate on the in vitro poly(A)-dependent synthesis of poly(Lys). There must therefore be a particular step or several steps in the elongation process in which the aminoacyl-tRNA either adopts an unknown intermediate structure, different from a 2′ or a 3′ isomer, or where the presence of the 2′-hydroxyl group is absolutely required.

An aminoacyl-tRNA exists in two isomeric forms. This is due to the fact that the aminoacyl residue can be attached to either the 2′ or the 3′ position of the terminal adenosine. Since the migration of the aminoacyl residue between the two vicinal *cis*-hydroxyl groups occurs spontaneously at standard physiological conditions (Griffin et al., 1966), there are potentially two substrates available for each particular step of the ribosomal peptide elongation process, namely, the 2′- or the 3′-aminoacyl-tRNA. The rate of isomerization exceeds the in vivo rate of the peptide bond formation by several orders of magnitude. It is therefore not possible to determine by direct chemical analysis which isomer of aminoacyl-tRNA is functional in the elongation process. To approach this problem, analogues of aminoacyl-tRNAs were prepared, in which the migration of the aminoacyl residue between the vicinal *cis*-hydroxyl groups is restricted by a chemical modification of the 3′-terminal adenosine of the tRNA. This can be achieved

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by cleavage of the C2′–C3′ bond of the ribose residue (Ofengand & Chen, 1972) or by the introduction of an amino group (Fraser & Rich, 1973).

The in vitro translation system, in which only the minimal necessary components are used, is generally limited to poly(U) or poly(A) as messengers. Therefore either $tRNA^{Lys}$ or $tRNA^{Phe}$ must be modified in order to be able to compare directly a pair of nonisomerizable aminoacyl-tRNAs. The site of aminoacylation on the tRNA is in most cases, however, very specific (Sprinzl & Cramer, 1974; Fraser & Rich, 1974; Chinault et al., 1977). A pair of aminoacylated deoxy-tRNA species could be prepared by enzymatic tyrosylation of $tRNA^{Tyr}$-CpCp2′dA[1] and $tRNA^{Tyr}$-CpCp3′dA (Sprinzl et al., 1977a), but the lack of the corresponding mRNA hindered the investigation of the activity of these tRNAs in the in vitro translation. Previous work on this field was therefore performed with Phe-$tRNA^{Phe}$-CpCp3′dA (Chinali et al., 1974; Baksht et al., 1976) and with aminoacylated tRNA fragments derived from the 3′ end of tRNA, which can be prepared by chemical synthesis (Bhuta et al., 1981; Krayevsky & Kukhanova, 1979, and references therein).

In this work we report on the preparation of Phe-$tRNA^{Lys}$-CpCp3′dA and Lys-$tRNA^{Lys}$-CpCp2′dA which allow a comparison of two isomeric aminoacyl-tRNA analogues with regard to their activity in the poly(A)-dependent *Escherichia coli* ribosomal system.

Experimental Procedures

Materials

Ribonuclease A from bovine pancreas (EC 3.1.27.5), snake venom phosphodiesterase (EC 3.1.4.1), and pyruvate kinase from rabbit muscle (EC 2.7.1.40), as well as unfractionated tRNA from *Escherichia coli* MRE 600, poly(A), ATP, GDP, GTP, GMPPCP, CTP, and phosphoenolpyruvate, were obtained from Boehringer (Mannheim, Germany). 3′-dATP was also a commercial product from Boehringer (Mannheim, Germany) and was purified by chromatography on DEAE-Sephadex A-25 prior to its use to assure the absence of ATP in the preparation. Sephadex A-25 and Sepharose 6B were from Pharmacia (Uppsala, Sweden), Bio-Gel P2 was from Bio-Rad (Richmond, CA), and AcA 44 was from LKB (Bromma, Sweden). Dithiothreitol and puromycin were purchased from Biomol (Ilvesheim, Germany).

Radioactively labeled [^{14}C]lysine and [^{14}C]phenylalanine with a specific activity of 50 Ci/mol were a product of Schwarz Radiochemicals (Orangeburg), and [^{14}C]phenylalanine (513 Ci/mol), [^{14}C]lysine (342 Ci/mol), [^{3}H]lysine (2.5 Ci/mmol), and [γ-^{32}P]GTP (13.9 Ci/mmol) were from Amersham-Buchler (Braunschweig, Germany). Whatman 3MM filter disks (Maidstone, Great Britain) and nitrocellulose filters (pore size 0.45 μm) from Millipore (Molsheim, France) were used. Aquasol scintillator fluid was from New England Nuclear (Boston, MA). Scintillation cocktail for counting in nonaqueous medium was prepared by dissolving 5.5 g of Scintimix III (Merck, Darmstadt, Germany) in 1 L of toluene.

ATP(CTP):tRNA nucleotidyltransferase from yeast (EC 2.7.7.25) with a specific activity of 44 000 units/mg of protein was a gift from Dr. H. Sternbach (Göttingen), and phenylalanyl-tRNA synthetase from yeast (EC 6.1.1.20) with a specific activity of 1800 units/mg of protein was supplied by Dr. F. von der Haar (Göttingen). Partially purified lysyl-tRNA synthetase from *E. coli* (EC 6.1.1.6) had a specific activity of 280 units/mg of protein and was prepared as described (Wagner & Sprinzl, 1979).

Elongation factors Tu and G were purified from an *E. coli* S-100 supernatant (Arai et al., 1972; Kaziro et al., 1972). EF-Tu·GDP with a specific activity of 19 000 units/mg of protein was kept frozen at −20 °C in a buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, and 10 μM GDP. EF-G with a specific activity of 3210 units/mg of protein (Rohrbach et al., 1974) was stored at −20 °C in a solution containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 0.25 M sucrose. Before use, it was extensively dialyzed against TMAKD buffer at 4 °C. Washed (1 M NH_4Cl) 70S ribosomes from *E. coli* were prepared as described by Gavrilova & Spirin (1974). The storage and the treatment before the tests were the same as in Wagner & Sprinzl (1980). Purified $tRNA^{Lys}$ was obtained after chromatography of bulk tRNA from *E. coli* according to the published procedure (Wagner & Sprinzl, 1980). For all ribosomal reactions, TMAKD buffer consisting of 60 mM Tris-HCl, pH 7.7, 30 mM KCl, 30 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM dithiothreitol was used.

Methods

Partial Enzymatic Hydrolysis of $tRNA^{Lys}$. $tRNA^{Lys}$ (100 A_{260} units) from *E. coli* was incubated with 5 μg/mL of snake venom phosphodiesterase in 1 mL of a solution containing 20 mM Tris-HCl, pH 7.6, and 5 mM $MgSO_4$ for 2 h at room temperature. The pH of the solution was adjusted to 5.2 by addition of 2 M sodium acetate, pH 4.5, and it was then applied onto a Sephadex A-25 column (1 × 3 cm) which had been equilibrated with 0.5 M NaCl and 20 mM sodium acetate, pH 5.2. After the column was washed with the same buffer, the $tRNA^{Lys}$-CpC was eluted by raising the concentration of NaCl in the buffer to 1 M. This tRNA-containing solution was desalted by filtration through a Bio-Gel P2 column (2.6 × 30 cm) and evaporated to dryness. The $tRNA^{Lys}$-CpC was redissolved in 1 mL of H_2O.

Incorporation of Deoxynucleotides into the 3′ End of $tRNA^{Lys}$-CpC. The reaction mixture (2 mL) containing 53 A_{260} units of $tRNA^{Lys}$-CpC, 2 μg of ATP(CTP):tRNA nucleotidyltransferase from yeast, 100 mM Tris-HCl, pH 9.0, 50 mM KCl, 10 mM $MgSO_4$, and either 4 mM 2′-dATP or 2 mM 3′-dATP was incubated for 2 h at room temperature. The $tRNA^{Lys}$ species were isolated by binding to Sephadex A-25, eluting with a buffer containing high salt concentration, and finally desalting on a Bio-Gel P2 column (Sprinzl et al., 1977b). Aqueous solutions (1 mL) of the obtained $tRNA^{Lys}$-CpCp2′dA and $tRNA^{Lys}$-CpCp3′dA were stored frozen at −20 °C.

Aminoacylation of $tRNA^{Lys}$ Species. Native $tRNA^{Lys}$ and the modified $tRNA^{Lys}$-CpCp2′dA were aminoacylated in a mixture containing 150 mM Tris-HCl, pH 7.7, 50 mM KCl, 10 mM $MgSO_4$, 5 mM dithiothreitol, 2 mM ATP, 0.02 mM [^{3}H]- or [^{14}C]lysine (the specific activity is indicated along with the single experiments), 3 μM of the respective $tRNA^{Lys}$ species, and 20 units/mL lysyl-tRNA synthetase from *E. coli*. Misaminoacylation of $tRNA^{Lys}$ and $tRNA^{Lys}$-CpCp3′dA with phenylalanine was achieved in a mixture containing 10 mM Tris-HCl, pH 9.0, 10 mM $MgSO_4$, 0.5 mM ATP, 0.02 mM [^{14}C]phenylalanine (specific activity as indicated in the experiments), 3 μM $tRNA^{Lys}$ species, and 500 units/mL phe-

[1] Abbreviations: EF-G and EF-Tu, *E. coli* elongation factors G and Tu; $tRNA^{Lys}$-CpCpA, native *E. coli* lysine-specific transfer RNA; $tRNA^{Lys}$-CpCp2′dA and $tRNA^{Lys}$-CpCp3′dA, $tRNA^{Lys}$ in which the 3′-terminal adenosine is replaced by 2′-deoxyadenosine or 3′-deoxyadenosine, respectively; Phe-$tRNA^{Lys}$, phenylalanyl-$tRNA^{Lys}$; Ac-Phe-$tRNA^{Lys}$, *N*-acetylphenylalanyl-$tRNA^{Lys}$; Ac_2-Lys-$tRNA^{Lys}$, N^{α},N^{ϵ}-diacetyllysyl-$tRNA^{Lys}$; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

nylalanyl-tRNA synthetase from yeast. The enzyme was incubated in 10 mM potassium phosphate, pH 7, and 10 mM EDTA for 15 min at 37 °C before it was used (Igloi et al., 1980). After incubation for 20 min at 37 °C, the solutions were extracted at 0 °C with the same volume of phenol which was saturated with 10 mM sodium acetate, pH 4.5. The aminoacyl-tRNA^{Lys} species in the aqueous phase were precipitated at −20 °C by the addition of 2.5 volumes of ethanol. After 2 h the precipitate was collected by centrifugation and washed once with 1 mL of 70% aqueous ethanol and twice with 1 mL of ethanol. The aminoacylated tRNAs were dried under vacuum in a desiccator and dissolved in 5 mM sodium acetate, pH 4.5. These solutions with a final concentration of about 10 μM aminoacyl-tRNA could be kept at −20 °C for a few weeks without appreciable loss of activity and amino acid hydrolysis. The extent of aminoacylation of all four tRNA^{Lys} species was generally 1200–1400 pmol of amino acid per A_{260} unit of tRNA.

N-Acetylation of Aminoacyl-tRNA^{Lys} Species. To 1 nmol of a [^{14}C]aminoacyl-tRNA^{Lys} in 0.1 mL of 5 mM sodium acetate, pH 4.5, was added 10 μL of 1 M sodium acetate, pH 5.2. This solution was mixed with 0.4 mL of *N*-acetoxysuccinimide (Rappoport & Lapidot, 1974), dissolved in freshly distilled dimethyl sulfoxide (75 mg/mL) and allowed to react for 2 h at 0 °C. The tRNA was precipitated by adding 0.5 mL of 10% aqueous dichloroacetic acid. After 1 h at 0 °C the acetylated aminoacyl-tRNA was pelleted by low-speed centrifugation, and the pellet was washed twice with 0.5 mL of ice-cold ethanol, then dried under vacuum, and redissolved in 5 mM sodium acetate, pH 4.5. The concentration of the *N*-acetylaminoacyl-tRNAs^{Lys} which were recovered in 80–90% yield was about 10 μM. The solutions were kept frozen at −20 °C.

As judged by thin-layer electrophoresis of an alkaline hydrolysate, the Phe-tRNA^{Lys} species were acetylated nearly quantitatively at the α-amino group of the amino acid. No more than 2–3% of free phenylalanine could be detected in the electropherogram after the acetylation reaction. In the case of the Lys-tRNA^{Lys} species, both the α- and the ε-amino groups were acetylated. This could be shown by the electrophoretic mobility of the products after alkaline hydrolysis. In addition about 10% of a monoacetylated derivative of the lysyl residue appeared in the electropherogram. Although this reaction product was not analyzed in detail, it was most likely the N^{α}-acetyllysine, since the α-amino group of lysine having a lower p*K* value than the ε-amino group (Greenstein & Winitz, 1961) is expected to react preferentially.

Thin-Layer Electrophoresis. Electrophoresis of *N*-acetylamino acids and peptides was carried out on cellulose thin-layer plates (20 × 20 cm) with 0.1 M ammonium acetate, pH 4.5. The electrophoretic separation was achieved by applying 450 V for 1 h. After the plates were dried, they were scanned for ^{14}C radioactivity on a Berthold thin-layer scanner.

Preparation of EF-Tu·GTP and EF-Tu·GMPPCP. EF-Tu·GDP was regenerated to EF-Tu·GTP or EF-Tu·GMPPCP in a reaction mixture containing 20 mM Tris-HCl, pH 7.8, 100 mM KCl, 10 mM $MgCl_2$, 4 mM phosphoenolpyruvate, 400 μg/mL pyurvate kinase, 0.8 mM GTP or 4 mM GMPPCP, and 20–30 μM EF-Tu·GDP. After 10 min at 37 °C, the mixture was chilled on ice and was kept at 0 °C up to 1 h before it was used.

Isolation of EF-Tu·[γ-^{32}P]GTP. In a total of 100 μL of a buffer containing 20 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 100 mM KCl, 30 nmol of EF-Tu·GDP (see above) was incubated with 20 μg of pyruvate kinase, 3.3 mM phosphoenolpyruvate, and 1.4 mM [γ-^{32}P]GTP for 10 min at 37 °C. The specific activity of the radioactive GTP was 300 Ci/mol. The mixture was cooled to 0 °C and injected onto an AcA 44 column (0.8 × 28 cm) equilibrated with 20 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 100 mM KCl at 4 °C. The material was eluted with the same buffer at 4 °C and collected in 0.45-mL fractions. The elution profile was monitored by determining the ^{32}P radioactivity in 20-μL aliquots. The fractions containing the highest concentration of EF-Tu·[γ-^{32}P]GTP (5.7 μM) were pooled and used directly for ribosomal binding experiments.

Measurement of the GTPase Activity. The hydrolyzed [^{32}P]phosphate was extracted from the reaction mixtures as phosphomolybdate (Martin & Doty, 1949). The reaction was stopped by the addition of 0.5 mL of a 20 mM silicotungstic acid solution in 0.02 N H_2SO_4, and then 0.1 mL of a 10 mM potassium phosphate buffer, pH 7.0, and 0.5 mL of 5% ammonium heptamolybdate in 4 N H_2SO_4 were added. After vigorous shaking the mixture was left for 5 min at room temperature and extracted with 2.5 mL of 2-methyl-1-propanol/benzene (1:1 v/v). The organic phase was removed and the ^{32}P radioactivity therein was determined by using Aquasol scintillation cocktail.

Ribosomal complexes with bound *N*-acetyl[^{14}C]aminoacyl-tRNA^{Lys} species were isolated by gel filtration on Sepharose 6B after EF-G promoted translocation. The *N*-acetyl[^{14}C]aminoacyl-tRNAs (specific activity 342 Ci/mol for lysine or 513 Ci/mol for phenylalanine) were bound to poly(A)-programmed ribosomes and subsequently translocated to the ribosomal P site by incubation with EF-G and GTP as described for Ac-[^{14}C]Phe-tRNA^{Lys} in the legend to Figure 2. The total volume of the reaction mixtures was 50 μL. The solutions were chilled and injected onto a Sepharose 6B column (0.8 × 19 cm) equilibrated with TMAKD buffer. The applied material was eluted at 4 °C into 0.17–0.18-mL fractions by using the same buffer. The elution profile was monitored by measuring 50-μL aliquots for ^{14}C radioactivity and by recording the absorbance at 260 nm. Ribosomal complexes appearing in the void volume of the column were used for testing the donor activity of the peptidyl-tRNA analogues.

Results

The measurements of the activities of nonisomerizable aminoacyl-tRNA analogues in single steps of the ribosomal elongation process are only meaningful if the preparations of these modified aminoacyl-tRNAs do not contain significant amounts of the native aminoacyl-tRNA. The removal of the 3′-terminal adenylate from tRNA^{Lys}-CpCpA was therefore performed with snake venom phosphodiesterase under carefully controlled conditions. A simple chromatographic method (Uziel et al., 1968; Sprinzl et al., 1977b) was used to monitor the rate of the AMP_{76} hydrolysis as well as the composition of the reaction products. Figure 1A shows the analysis of the tRNA^{Lys}-CpC preparation with regard to the 3′-terminal nucleosides in which about 5% tRNA^{Lys}-CpCpA is still present. This content of tRNA^{Lys}-CpCpA in the tRNA^{Lys}-CpC preparation also could be confirmed by the measurement of the enzymatic aminoacylation of tRNA^{Lys}-CpC in the presence and in the absence of AMP(CMP):tRNA nucleotidyltransferase (Sprinzl et al., 1977b). The experiments described below were set up in such a way that this contamination of native tRNA^{Lys} could not render results, the interpretation of which would be ambiguous. The incorporation of 2′-deoxyadenylate or 3′-deoxyadenylate into tRNA^{Lys}-CpC was accomplished with AMP(CMP):tRNA nucleotidyltransferase from yeast (Sprinzl & Sternbach, 1979) and yielded

Table I: Aminoacylation and Misaminoacylation of $tRNA^{Lys}$ Species from *E. coli*[a]

3′ terminus of the $tRNA^{Lys}$ species	specificity of the aminoacyl-tRNA synthetase	source	extent of aminoacylation (pmol/A_{260} unit of tRNA)	K_m (μM)	rel V_{max} (%)
-CpCpA	lysine	*E. coli*	1450	6.4	100
-CpCp2′dA	lysine	*E. coli*	1200	8.3	42
-CpCpA	phenylalanine	yeast	1520	5.6	100
-CpCp3′dA	phenylalanine	yeast	1220	2.1	29
-CpCp3′dA	lysine	*E. coli*	30	*b*	
-CpCp2′dA	phenylalanine	yeast	20		

[a] For aminoacylation the amino acid corresponding to the specificity of the aminoacyl-tRNA synthetase was used. Reaction conditions are described under Methods. [b] $K_i = 13.2$ μM.

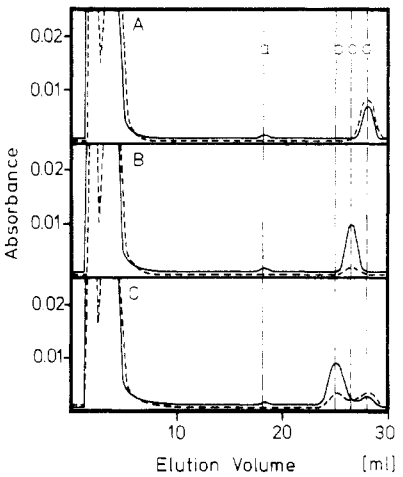

FIGURE 1: Analysis of the 3′-terminal nucleoside of the $tRNA^{Lys}$ species after digestion with pancreatic ribonuclease on a cation-exchange column according to Uziel et al. (1968). The 3′-terminal nucleosides are separated from the nucleotides and oligonucleotides which elute in the void volume. 1 A_{260} unit of the corresponding tRNA was digested with 25 μg of pancreatic ribonuclease in 100 mM phosphate buffer, pH 7, for 30 min at 37 °C. Samples (30–50 μL) were injected onto a column (0.6 × 20 cm) of Beckman M 71 ion-exchange resin and eluted with 0.4 M ammonium formiate, pH 4.5, at 50 °C. (A) $tRNA^{Lys}$-CpC, (B) $tRNA^{Lys}$-CpCp2′dA, and (C) $tRNA^{Lys}$-CpCp3′dA. The nucleosides to be detected elute as follows: (a) adenosine, (b) 3′-deoxyadenosine, (c) 2′-deoxyadenosine, and (d) cytidine.

Table II: Poly(A)-Directed Polypeptide Synthesis with Native and Modified Aminoacyl-$tRNA^{Lys}$ Species[a]

aminoacyl-$tRNA^{Lys}$	incorporation of amino acid into polypeptide (pmol/A_{260} unit of 70S ribosomes)		
	without elongation factors	with elongation factors	without poly(A)
Lys-$tRNA^{Lys}$	1.0	40.2	3.3
Lys-$tRNA^{Lys}$-CpCp2′dA	0.8	1.5	1.8
Phe-$tRNA^{Lys}$	1.2	64.9	9.0
Phe-$tRNA^{Lys}$-CpCp3′dA	0.5	1.7	1.2

[a] 0.72 A_{260} unit of 70S ribosomes was incubated at 37 °C for 30 min with 10 μg of poly(A), 28 μg of EF-Tu·GTP (for preparation, see Methods), 30 μg of EF-G, and 75 pmol of [^{14}C]aminoacyl-$tRNA^{Lys}$ (specific activity 342 Ci/mol for lysine or 513 Ci/mol for phenylalanine in TMAKD buffer). The total volume was 75 μL. For the determination of poly(Lys), samples were hydrolyzed in 1 M NaOH, neutralized with 1 M acetic acid, and filtered through nitrocellulose membranes after the addition of 2 mL of cold 0.25% sodium tungstate in 5% aqueous trichloroacetic acid according to Gardner et al. (1962). Poly(Phe) was precipitated on Whatman 3MM filters by hot 5% aqueous trichloroacetic acid (Erdmann et al., 1971).

$tRNA^{Lys}$-CpCp2′dA (Figure 1B) and $tRNA^{Lys}$-CpCp3′dA (Figure 1C), respectively. These modified $tRNAs^{Lys}$ contained less than 5% $tRNA^{Lys}$-CpCpA and no more regenerable $tRNA^{Lys}$-CpC. The small amount of $tRNA^{Lys}$-CpC (about 10%) which is present in the $tRNA^{Lys}$-CpCp2′dA preparation (Figure 1C) must be due to a contamination with inactivated $tRNA^{Lys}$ since repeated incubations of this tRNA species with the regenerating enzyme and ATP did not yield an increased amount of $tRNA^{Lys}$-CpCpA.

$tRNA^{Lys}$ from *E. coli* was aminoacylated with lysine by *E. coli* lysyl-tRNA synthetase (Table I). The misaminoacylation of this tRNA with phenylalanine is possible if the reaction is catalyzed by phenylalanyl-tRNA synthetase from yeast (Wagner & Sprinzl, 1980). The *E. coli* lysyl-tRNA synthetase has a 3′-hydroxyl-directed specificity (Sprinzl & Cramer, 1974) whereas the phenylalanyl-tRNA synthetase is 2′ specific (Sprinzl & Cramer, 1973). When a heterologous misaminoacylating system, $tRNA^{Lys}$ from *E. coli* and phenylalanyl-tRNA synthetase from yeast, was used, an attempt was made to show that the 2′ specificity of a synthetase can be transferred to a 3′-accepting tRNA. Data in Table I indicate that the phenylalanyl-tRNA synthetase retains its specificity and aminoacylates only $tRNA^{Lys}$-CpCp3′dA whereas $tRNA^{Lys}$-CpCp2′dA is not phenylalanylated. On the other hand lysyl-tRNA synthetase is able to attach the amino acid only to $tRNA^{Lys}$-CpCp2′dA but not to $tRNA^{Lys}$-CpCp3′dA. Thus a pair of nonisomerizable aminoacyl-$tRNA^{Lys}$ species becomes accessible for the study of the mechanism of ribosomal elongation, Phe-$tRNA^{Lys}$-CpCp3′dA and Lys-$tRNA^{Lys}$-CpCp2′dA.

The complex formation of the modified aminoacyl-$tRNA^{Lys}$ species with EF-Tu·GTP was investigated by a standard procedure (Beres & Lucas-Lenard, 1973; Pingoud et al., 1977; Wagner & Sprinzl, 1980). Both isomeric aminoacyl-tRNAs interact with the elongation factor as judged by the protection against hydrolysis of the amino acid ester linkage (data not shown). The ternary complex formed with Lys-$tRNA^{Lys}$-CpCp2′dA is apparently less stable than the one formed with Phe-$tRNA^{Lys}$-CpCp3′dA, but this difference should be mainly due to the fact that the phenylalanyl residue is preferentially bound as compared to the lysyl residue (Wagner & Sprinzl, 1980).

The activity of the nonisomerizable aminoacyl-$tRNA^{Lys}$ species during the translation was studied on poly(A)-programmed *E. coli* 70S ribosomes. The in vitro system used was strictly dependent on the presence of the elongation factors. As shown elsewhere (Wagner & Sprinzl, 1980; Pezzuto & Hecht, 1980) with a sufficient concentration of EF-Tu, the synthesis of poly(Phe) using Phe-$tRNA^{Lys}$ on poly(A)-programmed ribosomes is as efficient as the synthesis of poly(Lys) using Lys-$tRNA^{Lys}$ in this system. However, neither Phe-

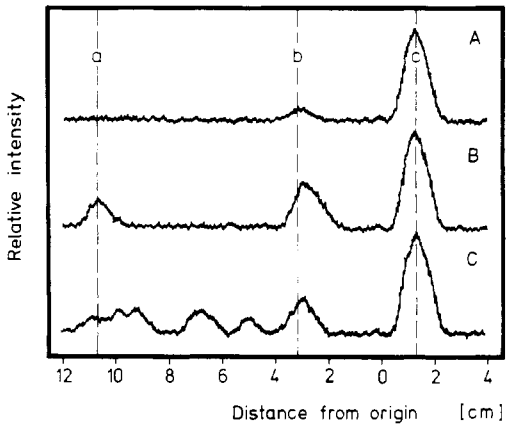

FIGURE 2: Thin-layer electrophoresis of the products of poly(A)-dependent polypeptide synthesis with native and modified Lys-tRNA^{Lys} species. 64 A_{260} units/mL 70S ribosomes, 1.0 mg/mL poly(A), and 2 μM *N*-Ac-[^{14}C]Phe-tRNA^{Lys} (specific activity 513 Ci/mol) were preincubated in TMAKD buffer for 20 min at 37 °C. Then 500 μg/mL EF-G and GTP up to a final concentration of 1 mM was added. Incubation was continued for 5 min at 37 °C, and the reaction mixture was chilled on ice. To 5 μL of a 6 μM [^{3}H]Lys-tRNA^{Lys} or [^{3}H]Lys-tRNA^{Lys}-CpCp2′dA (specific activity 2.5 Ci/mmol) solution in TMAKD buffer was added 5 μL of EF-Tu·GTP solution (prepared as described under Methods), and the mixture was kept at 0 °C for 2 min. To these solutions was added 20 μL of the preincubated ribosomes, and the reaction mixtures were incubated for 20 min at 37 °C. The reaction was stopped by the addition of 10 μL of 1 N acetic acid. The mixture was chilled, and the precipitated ribosomal complexes were concentrated by low-speed centrifugation. The pellets were hydrolyzed in 10 μL of 0.3 N NaOH for 30 min at 37 °C. After neutralization with 1 N acetic acid, an aliquot (about 6 μL) was applied to the thin-layer plate and subjected to electrophoresis as described under Methods. (A) Ac-[^{14}C]Phe-tRNA^{Lys} after alkaline hydrolysis, (B) products formed with [^{3}H]Lys-tRNA^{Lys}-CpCp2′dA, and (C) products formed with [^{3}H]Lys-tRNA^{Lys}. The positions of (a) Lysine, (b) phenylalanine, and (c) *N*-acetylphenylalanine in the electropherogram are indicated by the dashed lines.

tRNA^{Lys}-CpCp3′dA nor Lys-tRNA^{Lys}-CpCp2′dA is able to participate in polypeptide synthesis (Table II). Clearly, the absence of the vicinal hydroxyl group or the impediment of the compulsory migration of the aminoacyl residue between the two hydroxyl groups inhibits the incorporation of the amino acids into the growing peptide chain in the case of the nonisomerizable analogues of the aminoacyl-tRNA^{Lys}.

The electrophoretical analysis of the reaction products after polymerization on poly(A)-programmed ribosomes is shown in Figure 2. In this experiment the ribosomal P site was first saturated with a peptidyl-tRNA analogue, Ac-Phe-tRNA^{Lys} (Figure 2A), and then the reaction was started by the addition of elongation factors and of Lys-tRNA^{Lys}-CpCp2′dA or Lys-tRNA^{Lys}-CpCpA. In the case of native Lys-tRNA^{Lys}-CpCpA, as expected, a mixture of oligolysine molecules terminating with *N*-acetylphenylalanine was formed (Figure 2C). When Lys-tRNA^{Lys}-CpCp2′dA was used in this assay, only one discrete new oligopeptide migrating about 3 cm toward the anode could be detected (Figure 2B). Although the migration of this compound partly coincides with that of phenylalanine (Figure 2A), it is clear from the comparison of the ratio of peaks b and c in Figure 2A that a new oligopeptide was formed. Despite the fact that due to the lack of corresponding dipeptide standards, the new oligopeptide was not analyzed in detail, this result indicates that at least one peptide transfer must have occurred. Thus the 3′ isomer Lys-tRNA^{Lys}-CpCp2′dA is active as an acceptor of the peptidyl residue in the ribosomal A site.

The EF-Tu-dependent binding of the aminoacyl-tRNA^{Lys} species to poly(A)-programmed ribosomes was measured by the nitrocellulose filter assay. In the presence of a high excess

Table III: Binding of Aminoacyl-tRNA^{Lys} Species to Poly(A)-Programmed 70S Ribosomes at 0 °C[a]

aminoacyl-tRNA^{Lys}	binding (pmol of aminoacyl-tRNA/A_{260} unit of 70S ribosomes) without EF-Tu·GTP	with EF-Tu·GTP	stimulation	stimulation (% of total binding)
Lys-tRNA^{Lys}	3.1	9.9	6.8	69
Lys-tRNA^{Lys}-CpCp2′dA	3.1	7.8	4.7	60
Phe-tRNA^{Lys}	0.8	9.0	8.2	91
Phe-tRNA^{Lys}-CpCp3′dA	0.9	8.6	7.7	90

[a] 30 A_{260} units of 70S ribosomes, 1.0 mg/mL poly(A), and 10 A_{260} units/mL tRNA^{Lys} in TMAKD buffer were incubated for 10 min at 37 °C and then chilled on ice. Ternary complexes were prepared by adding 5 μL of [^{14}C]aminoacyl-tRNA^{Lys} in a TMAKD solution (3 μM) to 5 μL of EF-Tu·GTP (30 μM) as described in the legend to Figure 2. To this mixture 15 μL of the preincubated ribosomes was added, followed by incubation for 1 min at 0 °C. The samples were then diluted with ice-cold TMAKD buffer (2 mL), and the radioactivity retained on nitrocellulose membranes was determined according to the standard procedure (Wagner & Sprinzl, 1979). In the control experiments where EF-Tu·GTP was omitted, the corresponding amount of buffer was added.

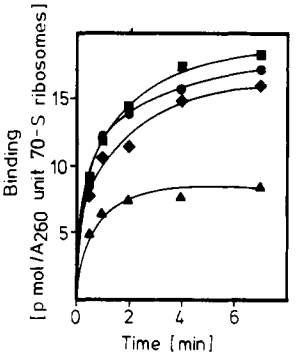

FIGURE 3: Time courses of the enzymatic binding of Phe-tRNA^{Lys} (♦), Lys-tRNA^{Lys} (●), Phe-tRNA-CpCp3′dA (■), and Lys-tRNA^{Lys}-CpCp2′dA (▲) to poly(A)-programmed 70S ribosomes in the presence of EF-Tu·GMPPCP. The binding reaction was performed as indicated in the footnote to Table III, but at 37 °C, 4-fold reaction mixtures were prepared, with the final concentrations being 0.5 μM for the aminoacyl-tRNAs^{Lys} and 3 μM for EF-Tu·GMPPCP. At appropriate time intervals, aliquots of 15 μL were withdrawm and diluted into 2 mL of cold TMAKD buffer. The binding was determined as described (Wagner & Sprinzl, 1979).

of uncharged tRNA, the enzymatic binding to the ribosomal A site can be measured (de Groot et al., 1971). The comparison of the Lys-tRNA^{Lys} and the Phe-tRNA^{Lys} species in this series of experiments is impaired by the fact that a lysylated tRNA^{Lys} under the chosen conditions (10 mM Mg^{2+}) shows a higher affinity to the ribosomes already in the absence of EF-Tu·GTP than a corresponding phenylalanylated tRNA species (Table III). Consequently the stimulation of the binding by EF-Tu·GTP, given as the difference of the values obtained in the presence of EF-Tu·GTP and in its absence, is lower for the Lys-tRNA^{Lys} species. The portion of the binding induced by the elongation factor is highest for Phe-tRNA^{Lys} with 91% and lowest for Lys-tRNA^{Lys}-CpCp2′dA with 60%. However, these experiments could not provide conclusive information about the precise location of the aminoacyl residue of aminoacyl-tRNA^{Lys} species on the ribosomes during the binding process. The interaction of the particular ternary complexes with programmed 70S ribosomes was therefore studied by measuring the time course of the binding in the presence of EF-Tu·GMPPCP (Figure 3). This

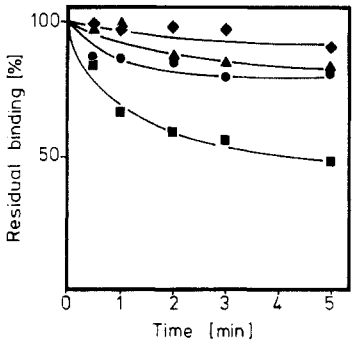

FIGURE 4: Displacement of Lys-tRNA^{Lys} (●), Phe-tRNA^{Lys} (♦), Lys-tRNA^{Lys}-CpCp2′dA (▲), and Phe-tRNA^{Lys}-CpCp3′dA (■), bound enzymatically to poly(A)-programmed 70S ribosomes, by Lys-tRNA^{Lys}. The aminoacyl-tRNA^{Lys} species were bound to the ribosomes in the presence of EF-Tu·GTP as indicated in the legend to Figure 3. After incubation for 7 min at 37 °C, [^{14}C]Lys-tRNA^{Lys} (specific activity 50 Ci/mol) was added. The final concentration of the native Lys-tRNA^{Lys} was 0.5 μM, and the total volume of the reaction mixtures was 110 μL. The residual binding of the individual aminoacyl-tRNAs^{Lys} was determined as indicated in the footnote to Table III upon the removal of 20-μL aliquots at definite time intervals. The values were not corrected for successive binding of the equimolar amount of [^{14}C]Lys-tRNA^{Lys} which was added, since its specific activity was only one-tenth of the specific activity of the aminoacyl-tRNA^{Lys} species originally bound to the ribosomes.

nonhydrolyzable GTP analogue should not interfere with the binding reaction, but when the GTPase activity of EF-Tu is blocked, the following steps after the binding process should be impeded (Girbes et al., 1976). Under these conditions Phe-tRNA^{Lys}-CpCp3′dA has the same binding activity as the two native species, Phe-tRNA^{Lys} and Lys-tRNA^{Lys}. The ability of Lys-tRNA^{Lys}-CpCp2′dA to bind to the ribosomes is on the other hand severely impaired (Figure 3).

Although Phe-tRNA^{Lys}-CpCp3′dA, where the aminoacyl residue is localized at the 2′ position, is more active in the binding reaction than the corresponding 3′ analogue, Lys-tRNA^{Lys}-CpCp2′dA (Figure 3), its interaction with the ribosomal A site is weaker. From all four aminoacyl-tRNAs investigated, only the prebound Phe-tRNA^{Lys}-CpCp3′dA can be exchanged to a significant extent by an equimolar amount of Lys-tRNA^{Lys} (Figure 4). These experiments were performed in the presence of EF-Tu·GTP; i.e., the prebound aminoacyl-tRNAs should be located in the correct final position of the ribosomal A site (Girbes et al., 1976). Evidently, since Phe-tRNA^{Lys}-CpCp3′dA can be displaced to a high extent, it is not accommodated properly into the binding site on the 70S ribosome.

The process of EF-Tu-mediated binding of the aminoacyl-tRNA to the ribosome is accompanied by a hydrolysis of GTP (Miller & Weissbach, 1977) in which one molecule of GTP is hydrolyzed to GDP for each molecule of aminoacyl-tRNA bound. Data on the GTPase activity associated with the binding of the different aminoacyl-tRNAs^{Lys} are summarized in Table IV. Whereas Phe-tRNA^{Lys}, Lys-tRNA^{Lys}, and Lys-tRNA^{Lys}-CpCp2′dA give rise to about the same stoichiometry of GTP hydrolyzed per aminoacyl-tRNA bound, Phe-tRNA^{Lys}-CpCp3′dA shows a significantly increased value. The higher GTPase activity in the case of Phe-tRNA^{Lys}-CpCp3′dA can be connected to its loose interaction with the ribosomal A site. Since this aminoacyl-tRNA, once bound to the ribosomal A site, is readily exchanged by a ternary complex present in solution (Figure 4), each displacement will lead to a new GTP hydrolysis. This could explain the observed increase in the GTPase activity during the EF-Tu·GTP-dependent binding of Phe-tRNA^{Lys}-CpCp3′dA.

Table IV: GTP Hydrolysis upon Enzymatic Binding of Aminoacyl-tRNA^{Lys} Species to Poly(A)-Programmed 70S Ribosomes[a]

aminoacyl-tRNA^{Lys}	[^{32}P]GTP hydrolyzed (pmol/A_{260} unit of 70S ribosomes)	GTP hydrolyzed/ aminoacyl-tRNA bound
Lys-tRNA^{Lys}	7.5	0.87
Lys-tRNA^{Lys}-CpCp2′dA	7.9	0.88
Phe-tRNA^{Lys}	6.3	0.81
Phe-tRNA^{Lys}-CpCp3′dA	9.7	1.45

[a] The preincubation mixture contained 75 A_{260} units/mL 70S ribosomes, 1.7 mg/mL poly(A), and 17 A_{260} units/mL tRNA^{Lys} in TMAKD buffer. After 10 min at 37 °C, 10 μL of this solution was added at 0 °C to a mixture of 5 μL of [^{14}C]aminoacyl-tRNA^{Lys} (4 μM in TMAKD buffer; specific activity 342 Ci/mol for lysine or 513 Ci/mol for phenylalanine) and 25 μL of 5.7 μM EF-Tu·[γ-^{32}P]GTP, isolated as described under Methods. The reaction was allowed to proceed for 1 min at 0 °C; then a 20-μL aliquot was withdrawn and analyzed for GTP hydrolysis (see Methods). The remaining reaction solution was used to determine the binding of the aminoacyl-tRNAs to the ribosomes by the standard method given in the footnote to Table III. The values were corrected for the amount of GTP hydrolyzed in the absence of ribosomes.

Table V: Puromycin Reaction with *N*-Acetylaminoacyl-tRNA^{Lys} Species Bound to Poly(A)-Programmed 70S Ribosomes[a]

N-acetylaminoacyl-tRNA^{Lys}	product formation with puromycin (% of *N*-acetylaminoacyl-tRNA^{Lys} species bound to ribosomes)	
	without EF-G	with EF-G
Ac_2-Lys-tRNA^{Lys}	7	27
Ac_2-Lys-tRNA^{Lys}-CpCp2′dA	40	38
Ac-Phe-tRNA^{Lys}	26	63
Ac-Phe-tRNA^{Lys}-CpCp3′dA	6	8

[a] Reaction mixtures containing 40 A_{260} units/mL 70S ribosomes, 1.3 mg/mL poly(A), and 1.5 μM *N*-acetyl[^{14}C]aminoacyl-tRNA^{Lys} (specific activity 342 Ci/mol for lysine or 513 Ci/mol for phenylalanine) species in 60 μL of TMAKD buffer were prepared and incubated for 15 min at 37 °C. To half of these solutions were added EF-G and GTP at 0 °C to final concentrations of 1 mg/mL and 1 mM, respectively. The other half was diluted with a corresponding amount of TMAKD buffer. These reaction mixtures with a total volume of 38 μL each were further incubated for 5 min at 37 °C. Then 18 μL of a sample which contained EF-G and 18 μL of one without EF-G were allowed to react with puromycin for 10 min at 37 °C upon the addition of 2 μL of a 10 mM aqueous solution of the antibiotic. The extent of puromycin peptide formation was then determined by diluting with 1 mL of 100 mM sodium acetate, pH 5.2, and extracting this solution with 1.5 mL of ethyl acetate at room temperature (Leder & Bursztyn, 1966). The radioactivity in the organic phase was measured after the addition of Aquasol. An identical pair of samples was diluted with 2 μL of water, incubated under the same conditions, and used to determine the binding of the *N*-acetyl-[^{14}C]aminoacyl-tRNAs to the ribosomes by the usual procedure.

The ability of peptidyl-tRNA analogues derived from Lys-tRNA^{Lys}, Phe-tRNA^{Lys}, Lys-tRNA^{Lys}-CpCp2′dA, and Phe-tRNA^{Lys}-CpCp3′dA to translocate from the ribosomal A site to the ribosomal P site and to react subsequently with an acceptor molecule bound to the ribosomal A site was measured by the puromycin reaction. In the experiments summarized in Table V the respective *N*-acetylaminoacyl-tRNAs were bound to 70S ribosomes, and the formation of

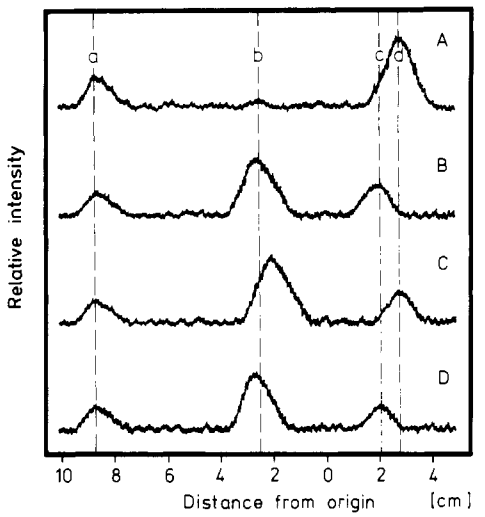

FIGURE 5: Thin-layer electrophoresis of the products formed between acetyl[^{14}C]aminoacyl-tRNA^{Lys} species bound to poly(A)-programmed ribosomes, after EF-G promoted translocation and [^{3}H]Lys-tRNA^{Lys} bound to these ribosomes in the presence of EF-Tu·GTP. The ribosomal complexes with bound *N*-acetylaminoacyl-tRNA^{Lys} species were prepared and subsequently isolated by gel filtration on Sepharose 6B as indicated under Methods. The fractions containing the major amount of *N*-acetylaminoacyl-tRNA complexed to the ribosomes were pooled. The concentration of the ribosome-bound *N*-acetyl[^{14}C]-aminoacyl-tRNA in the joined fractions was determined to be 35–50 nM. To 500 μL of these solutions was added a mixture of 10 μL of [^{3}H]Lys-tRNA^{Lys} (4 μM in TMAKD; specific activity 2.5 Ci/mmol) and 10 μL of EF-Tu·GTP (30 μM; see Methods). The samples were incubated at 37 °C for 10 min and chilled, and 10 μL of glacial acetic acid was added. The precipitate was pelleted and hydrolyzed with 0.3 N NaOH, and aliquots were subjected to thin-layer electrophoresis as described in the legend to Figure 2. Products were formed with Ac_2-[^{14}C]Lys-tRNA^{Lys} (D), Ac-[^{14}C]Phe-tRNA^{Lys} (C), Ac_2-[^{14}C]-Lys-tRNA^{Lys}-CpCp2′dA (B), and Ac-[^{14}C]Phe-tRNA^{Lys}-CpCp3′dA (A). The migration distances of (a) lysine, (b) phenylalanine, (c) N^{α},N^{ϵ}-diacetyllysine, and (d) *N*-acetylphenylalanine are indicated by the vertical broken lines.

the new peptide bond was measured in the presence and in the absence of elongation factor G after the addition of puromycin. Ac_2-Lys-tRNA^{Lys}-CpCp2′dA is able to function as a donor of the *N*-acetylaminoacyl residue whereas the Ac-Phe-tRNA^{Lys}-CpCp3′dA is inactive in this reaction. Furthermore it can be seen from the data in Table V that only in the case of the *N*-acetylaminoacyl-tRNAs^{Lys} derived from the native tRNA^{Lys}, a stimulation of the peptidyl transfer to puromycin occurs in the presence of EF-G. Apparently the binding of Ac_2-Lys-tRNA^{Lys}-CpCp2′dA is more selectively directed to the ribosomal P site, and therefore no stimulation of the peptide formation can be observed after the addition of EF-G. More direct evidence showing the specificity of the ribosomal donor site for the 3′-peptidyl isomer is shown in Figure 5. In these experiments the dipeptide formation was measured on ribosomes in which the P site was occupied by a peptidyl-tRNA or its nonisomerizable analogue and the A site was occupied by an aminoacyl-tRNA. The products were hydrolyzed and analyzed by thin-layer electrophoresis. In order to remove the excess ^{14}C-labeled *N*-acetylaminoacyl-tRNA species from the complexes of the peptidyl-tRNA analogues with programmed 70S ribosomes, the incubation mixtures were subjected to gel filtration on a Sepharose 6B column. During this step also the elongation factor G, used to translocate the peptidyl-tRNA to the ribosomal P site, was separated from the complexes. In the absence of elongation factor G the formation of a single dipeptide should occur. This was the case for the ribosomal complexes with Ac-Phe-tRNA^{Lys}, Ac_2-Lys-tRNA^{Lys}, and Ac_2-Lys-tRNA^{Lys}-CpCp2′dA bound to the donor site (Figure 5), where a new ^{14}C-labeled oligopeptide appears at 2–3-cm distance from the origin toward the anode. If Ac-Phe-tRNA^{Lys}-CpCp3′dA was prebound to the ribosomes, no dipeptide formation took place.

Discussion

The aim of this work was to elucidate the ability of nonisomerizable aminoacyl-tRNA species to participate in the particular steps of the translation process, using an in vitro translation system derived from *E. coli*. Since two isomeric deoxy-tRNAs^{Lys} were used in our investigations, the role of the missing hydroxyl group during peptide transfer, translocation, and binding of aminoacyl-tRNA could also be approached in this study. The results presented here are an extension of experiments performed previously and reported by Chinali et al. (1974) and Baksht et al. (1976). In contrast to the previous studies where heterologous in vitro systems in which the components were derived from different organisms were used, in this paper experiments are described which were performed in a homologous *E. coli* system.

The preparation of Lys-tRNA^{Lys}-CpCp2′dA and Phe-tRNA^{Lys}-CpCp3′dA was accomplished by standard methods described previously (Sprinzl et al., 1977b; Wagner & Sprinzl, 1979). With respect to the specificity of the aminoacylation reaction at the 2′- or the 3′-hydroxyl group of the terminal adenosine of tRNA, the question has arisen whether this specificity is determined by the tRNA structure or if it is an intrinsic property of the catalytic activity of the particular synthetase (Sprinzl & Cramer, 1979). Since the 2′ or 3′ specificity during the aminoacylation may play a role in the mechanism of proofreading processes (von der Haar & Cramer, 1976; Igloi et al., 1978), the answer to this question has important implications. Using the misaminoacylation assay in which tRNA^{Lys} from *E. coli* is aminoacylated by phenylalanyl-tRNA synthetase from yeast (Alford & Hecht, 1978; Wagner & Sprinzl, 1980; Pezzuto & Hecht, 1980), we could approach this problem experimentally. From the pair of tRNA^{Lys}-CpCp2′dA and tRNA^{Lys}-CpCp3′dA in a lysyl-tRNA synthetase catalyzed reaction, only the tRNA possessing the terminal 2′-deoxyadenosine could be aminoacylated. Conversely, the phenylalanyl-tRNA synthetase phenylalanylates only the tRNA with a terminal 3′-deoxyadenosine, disregarding the specificity of the tRNA molecule. These results are in agreement with the recently reported observations of Alford & Hecht (1978) and show that the phenylalanyl-tRNA synthetase is the component which determines the 2′-directed aminoacylation of tRNA^{Phe} (Sprinzl & Cramer, 1973). This further suggests that the aminoacylation and misaminoacylation are closely related with respect to their reaction mechanism. Alford & Hecht (1978), who measured the kinetics of the aminoacylation of the isomeric deoxy-tRNAs^{Ala} from *E. coli* with yeast phenylalanyl-tRNA synthetase, obtained similar results. Therefore it appears that the site of primary attachment of the amino acid to the tRNA is directed by the architecture of the active site of the synthetase and not by the structure of the tRNA. In those cases, however, where both deoxy-tRNA isomers are able to accept the amino acid, although with a different rate and to a different extent, this experimental approach may be misleading. It was reported by Alford & Hecht (1979) that tRNA^{Trp} from *E. coli* is activated preferentially on the 2′-hydroxyl group by both yeast and *E. coli* tryptophanyl-tRNA synthetase, whereas the yeast tRNA^{Trp} is activated preferentially on its 3′-hydroxyl group by both enzymes. As pointed out in the above report, this may be due to the change in the balance between the enzymatic aminoacylation and deacylation

when deoxy-tRNAs^{Trp} from different sources are used in the in vitro aminoacylation assay.

Both, nonisomerizable aminoacyl-tRNA species, Phe-tRNA^{Lys}-CpCp3′dA and Lys-tRNA^{Lys}-CpCp2′dA, form ternary complexes with EF-Tu·GTP. This result is in agreement with previous reports (Sprinzl et al., 1977a,b; Alford et al., 1979). We observed a pronounced preference of EF-Tu·GTP for Phe-tRNA^{Lys}-CpCp3′dA as compared to Lys-tRNA^{Lys}-CpCp2′dA. However, the preference for the 2′-isomeric aminoacyl-tRNA is probably not due to the location of the amino acid at the 2′ position of the terminal adenosine but rather reflects the different affinities of Phe-tRNA^{Lys} and Lys-tRNA^{Lys} to EF-Tu·GTP. It could be shown by independent investigations that the aminoacyl residue of the charged tRNA contributes significantly to the stability of ternary complexes (Sprinzl & Wagner, 1980; Pingoud & Urbanke, 1980). Furthermore it was clearly demonstrated by Alford et al. (1979) that the *E. coli* EF-Tu does not exhibit a general preference for one of the two aminoacyldeoxy-tRNAs. Since the aminoacyldeoxy-tRNA species, regardless of the position to which the amino acid is attached, always interact more weakly with the EF-Tu as the corresponding native aminoacyl-tRNA (Alford et al., 1979), it appears that the native CpCpA terminus of the aminoacyl-tRNA with its mobile aminoacyl residue is a prerequisite for an efficient interaction.

The EF-Tu-dependent binding of Phe-tRNA^{Lys}-CpCp3′dA to programmed ribosomes is as efficient as that of the aminoacyl-tRNAs with an intact CpCpA end. The binding of Lys-tRNA^{Lys}-CpCp2′dA is also stimulated by the presence of the elongation factor Tu. However, the extent of binding in this case does not reach the level of the native Lys-tRNA^{Lys}-CpCpA. It is also remarkable that Lys-tRNA^{Lys}-CpCp2′dA in contrast to Phe-tRNA^{Lys}-CpCp3′dA binds nonenzymatically very efficiently to poly(A)-programmed ribosomes. This result agrees with the observation of Fraser & Rich (1973) and of Baksht et al. (1976), who showed that Phe-tRNA^{Phe}-CpCp3′NH_2A, although not active in the aminoacyl-tRNA·EF-Tu·GTP ternary complex formation, is able to bind to the ribosomal A site with increased magnesium concentration very efficiently and to a higher extent than the native Phe-tRNA^{Phe}-CpCpA.

Both nonisomerizable aminoacyl-tRNAs give rise to GTP hydrolysis in the process of EF-Tu·GTP-dependent binding. The main difference between both isomeric aminoacyl-tRNAs in this process concerns the stoichiometry of the GTP hydrolysis. In the case of Phe-tRNA^{Lys}-CpCp3′dA, the formation of GDP is significantly higher than the stoichiometrically expected amount. This finding can be interpreted in connection with other observations reported in this work, namely, that Phe-tRNA^{Phe}-CpCp3′dA once bound to ribosomes can be exchanged by another native aminoacyl-tRNA species. From this follows that Phe-tRNA^{Lys}-CpCp3′dA bound to the ribosomal A site does not occupy its final location and can still dissociate. This obviously gives rise to the binding of a new ternary complex with consecutive GTP hydrolysis. From the presented experiments it is concluded that both isomers of aminoacyl-tRNAs are able to interact with the ribosomal A site in the EF-Tu-dependent process. The 2′-aminoacyl-tRNA species is more active in this reaction, whereas the 3′-aminoacyl-tRNA species is more tightly bound to the final locus of the aminoacyl-tRNA on the ribosomes. The latter position probably corresponds to the location of native aminoacyl-tRNA after GTP hydrolysis and EF-Tu·GDP release.

Table VI: Activity of Nonisomerizable Aminoacyl-tRNA Analogues in Distinct Steps during Ribosomal Peptide Chain Elongation

step	activity of 2′ isomer	activity of 3′ isomer
aminoacyl-tRNA·EF-Tu·GTP ternary complex formation	+[a]	+[a]
binding of ternary complex to the ribosomal A site	+[b]	+
acceptor activity	+[c]	+
binding of the *N*-acetyl derivative to the ribosomal P site	+	+
donor activity of the *N*-acetyl derivative	−	+

[a] Interaction with EF-Tu·GTP is weaker than in the case of unmodified aminoacyl-tRNAs. [b] Can readily dissociate from the ribosomal A site in the presence of native aminoacyl-tRNA. [c] Very slow formation of dipeptide.

Only the aminoacyl-tRNA which has an aminoacyl residue on the 3′ position of the terminal adenosine is competent as an efficient acceptor of the peptidyl residue. This finding agrees well with investigations performed with aminoacyl-tRNA fragments (Chladek et al., 1974; Ringer et al., 1975; Krayevsky & Kukhanova, 1980; Bhuta et al., 1981) or with puromycin analogues (Nathans & Neidle, 1963). The formation of the peptide bond with a 2′-aminoacylated tRNA species, although measurable in an in vitro assay (Chinali et al., 1974), proceeds with a rate which is too slow to account for the speed of the in vivo translation process.

From the investigation of the activity of Ac-Phe-tRNA^{Phe}-CpCp3′dA as a peptide donor, Chinali et al. (1974) concluded either that the 2′-peptidyl-tRNA is the wrong isomer to donate the peptidyl residue or that the vicinal 3′-hydroxyl group is necessary for the peptidyl transfer reaction. In this work we could demonstrate that the presence of the vicinal hydroxyl group on the terminal adenosine is not an absolute prerequisite for the transfer of the peptidyl residue from the 3′ position of the peptidyl-tRNA, since the Ac-Lys-tRNA^{Lys}-CpCp2′dA is active as a donor. On the other hand, the peptidyl-tRNA^{Lys}-CpCp3′dA, in agreement with the previous work (Chinali et al., 1974), is inactive in this reaction. Although we did not present data that show the binding of Ac-Phe-tRNA^{Lys}-CpCp3′dA to the ribosomal P site, the evidence that a similar analogue of peptidyl-tRNA, Ac-Phe-tRNA^{Phe}-CpCp3′dA, is able to interact efficiently with this site was brought in our previous work (Chinali et al., 1974; Baksht et al., 1976). It can therefore be concluded that although both isomers of peptidyl-tRNA are able to interact with the P site of ribosomes only the species in which the peptidyl residue is bound to the 3′ position is competent in the peptide transfer.

The results on the 2′ and 3′ specificity of the ribosomal system for an aminoacyl-tRNA during the particular steps of the peptide elongation process are summarized in Table VI. The tRNA in which the aminoacyl or peptidyl residue is bound to the 3′ position of the terminal adenosine is active in all isolated steps of elongation, which can be investigated separately. Nevertheless, this nonisomerizable aminoacyl- (or peptidyl-) tRNA is not able to participate in the overall polypeptide synthesis. This implies that there must be a particular step, or several steps in the elongation cycle, not considered in this work, in which the aminoacyl-tRNA either adopts an unknown intermediate structure or where the presence of the 2′-hydroxyl group is absolutely required. The evidence that this step does not only concern the translocation

of the peptidyl-tRNA from the A to the P site will be the subject of a forthcoming communication.

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