Inhibition of Ribosomal Translocation by Peptidyl Transfer Ribonucleic Acid Analogues†

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ABSTRACT: The activity of peptidyl-tRNA^{Lys}-CpCp2′dA was measured in an in vitro poly(A)-dependent polypeptide synthesizing system derived from *Escherichia coli*. It has already been shown that Lys-tRNA^{Lys}-CpCp2′dA is active as an acceptor and Ac_2-Lys-tRNA^{Lys}-CpCp2′dA can donate its peptidyl residue but that the overall poly(A)-dependent synthesis of polylysine does not take place with Lys-tRNA^{Lys}-CpCp2′dA [Wagner, T., Cramer, F., & Sprinzl, M. (1982) *Biochemistry 21*, 1521-1529]. This is due to the efficient inhibition of the EF-G-dependent translocation of the peptidyl-tRNA-CpCp2′dA from the ribosomal A to the ribosomal P site. In addition, the EF-G-dependent release of the deacylated tRNA^{Lys}-CpCp2′dA from the ribosomes is also inhibited. The action of the elongation factor G or some other ribosomal component participating in the translocation process requires the presence of the 2′-hydroxyl group on the terminal adenosine of tRNA. If this hydroxyl group is not present on the tRNA, the ribosomes remain locked in their pretranslocational state.

Modified aminoacyl-tRNAs, in which the 3′-terminal adenosine of the polynucleotide chain is replaced by 2′-deoxyadenosine or 3′-deoxyadenosine, have been prepared (Sprinzl et al., 1973; Alford et al., 1979). In these species the migration of the aminoacyl residue is hindered by the absence of the vicinal hydroxyl group (Sprinzl & Cramer, 1973). These analogues were widely used in the past to unravel the possible role of the fast migration of the acyl residue between the 2′ and the 3′ position on the native aminoacyl- or peptidyl-tRNA during the ribosomal polypeptide elongation (Sprinzl & Cramer, 1979; Hecht, 1977). In the previous communication we described the preparation of Phe-tRNA^{Lys}-CpCp3′dA[1] and Lys-tRNA^{Lys}-CpCp2′dA, a pair of nonisomerizable aminoacyl-tRNA analogues, which were investigated in a poly(A)-dependent ribosomal translation system derived from *Escherichia coli* (Wagner et al., 1982). Both modified aminoacyl-tRNAs were active in the nonenzymatic or EF-Tu-dependent binding to 70S ribosomes, although with different efficiency. The 3′-aminoacylated Lys-tRNA^{Lys}-CpCp2′dA was also active as an acceptor of the peptidyl residue and Ac_2-Lys-tRNA^{Lys}-CpCp2′dA did function as a donor. The isomeric aminoacyl- and peptidyl-tRNAs derived from tRNA^{Lys}-CpCp3′dA were not active in these processes. Despite its ability to participate in the peptidyl transfer reactions, Lys-tRNA^{Lys}-CpCp2′dA was inactive in the poly(A)-dependent synthesis of polylysine, indicating that the EF-G-dependent translocation process is inhibited by the 2′-deoxy analogues.

In the present work the activity of peptidyl-tRNA^{Lys}-CpCp2′dA in the individual translocation reactions was investigated.

Materials and Methods

Snake venom phosphodiesterase (EC 3.1.4.1) and pyruvate kinase from rabbit muscle (EC 2.7.2.40) as well as poly(A), GTP, 2′dATP and phosphoenolpyruvate were obtained from Boehringer (Mannheim, West Germany). 3′dATP was prepared by phosphorylating 3′-deoxyadenosine (Sigma, St. Louis, MO) according to established procedures (Hoard & Ott, 1965; Yoshikawa et al., 1967). Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden). Polygram Cel 400 thin-layer sheets from Macherey, Nagel and Co. (Düren, West Germany) were used for electrophoresis. Analytical-grade chemicals were from Merck (Darmstadt, West Germany).

[^{14}C]Lysine (specific activity 50 Ci/mol) was a product of Schwarz Radiochemicals (Orangeburg, NY). [^{14}C]Phenylalanine (513 Ci/mol), [^{3}H]phenylalanine (80 Ci/mmol), [^{14}C]lysine (342 Ci/mol), [^{3}H]lysine (40 Ci/mmol), and [^{3}H]2′dATP (10 Ci/mmol) were obtained from Amersham Buchler (Braunschweig, West Germany). The radioactivity in aqueous samples were measured after the addition of Aquasol scintillation fluid from New England Nuclear (Boston, MA). A scintillation cocktail, prepared by dissolving 5.5 g of Scintimix III (Merck, Darmstadt, West Germany) in 1 L of toluene was used to measure the radioactivity of the precipitates placed on filter disks. The specific activity of the radioactive compounds for double-labeling experiments was adjusted as described in the legend to the figures.

ATP(CTP):tRNA nucleotidyltransferase from yeast (EC 2.7.7.25) with a specific activity of 44 000 units/mg of protein (Sternbach et al., 1971) was obtained from Dr. H. Sternbach (Göttingen); phenylalanyl-tRNA synthetase from yeast (EC 6.1.1.20) with a specific activity of 1800 units/mg of protein (von der Haar, 1973) was supplied by Dr. F. von der Haar (Göttingen). Partially purified lysyl-tRNA synthetase from *E. coli* (EC 6.1.1.6) with a specific activity of 280 units/mg of protein was prepared as described (Wagner & Sprinzl, 1979).

Elongation factors Tu and G from *E. coli* were purified according to Arai et al. (1972) and Kaziro et al. (1972), respectively. These factors had specific activities of 19 000 units/mg (EF-Tu) and 3210 units/mg (EF-G) and were treated as described (Wagner & Sprinzl, 1982). Washed 70S

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[1] Abbreviations: EF-G and EF-Tu, *Escherichia 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 and 3′-deoxyadenosine, respectively; Phe-tRNA^{Lys}, phenylalanyl-tRNA^{Lys}; Ac-Phe-tRNA^{Lys}, *N*-acetylphenylalanyl-tRNA^{Lys}; Ac_2-Lys-tRNA^{Lys}, N,N^{ϵ}-diacetyllysyl-tRNA^{Lys}; Tris, tris(hydroxymethyl)aminomethane.

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ribosomes from *E. coli* were prepared, stored, and treated before being used in the single experiments, as published (Wagner & Sprinzl, 1979, and references cited therein). $tRNA^{Lys}$-CpCpA was isolated from *E. coli* bulk tRNA (Boehringer, Mannheim, West Germany) by the procedure previously described (Wagner & Sprinzl, 1980). For all 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.

$tRNA^{Lys}$-CpC was prepared by partial enzymatic hydrolysis of $tRNA^{Lys}$-CpCpA with snake venom phosphodiesterase according to Sprinzl et al. (1972). $tRNA^{Lys}$-CpCp2′dA, $tRNA^{Lys}$-CpCp3′dA, and $tRNA^{Lys}$-CpCp[^{3}H]2′dA (specific activity 200 Ci/mol) were obtained by incorporating the corresponding modified and radioactively labeled adenylates into the 3′ end of $tRNA^{Lys}$-CpC by using ATP(CTP):tRNA nucleotidyltransferase as described by Sprinzl & Sternbach (1979). The integrity of the modified $tRNA^{Lys}$ species could be confirmed after the analysis of the 3′-terminal nucleoside by ion-exchange column chromatography (Sprinzl et al., 1977; Wagner et al., 1982). $tRNA^{Lys}$-CpCpA was aminoacylated to an extent of 1450 pmol/A_{260} unit of tRNA with lysine or with phenylalanine by using Lys-tRNA synthetase from *E. coli* or Phe-tRNA synthetase from yeast, respectively. $tRNA^{Lys}$-CpCp2′dA and $tRNA^{Lys}$-CpCp[^{3}H]2′dA were lysylated with Lys-tRNA synthetase from *E. coli* to an extent of 1200 pmol of lysine/A_{260} unit of tRNA. Misaminoacylation of $tRNA^{Lys}$-CpCp3′dA with ^{14}C-labeled phenylalanine was achieved with phenylalanyl-tRNA synthetase from yeast to an extent of 1220 pmol of phenylalanine/A_{260} unit of tRNA. The conditions for the enzymatic aminoacylation reactions are described in Wagner et al. (1982). The specific activities of the radioactively labeled aminoacyl-tRNAs are indicated in the legends to the figures. The primary amino groups of the ^{14}C-labeled amino acids of Lys-$tRNA^{Lys}$, Lys-$tRNA^{Lys}$-CpCp2′dA, Phe-$tRNA^{Lys}$, and Phe-$tRNA^{Lys}$-CpCp3′dA were N-acetylated by the method of Rappoport & Lapidot (1974). The reaction products were analyzed by thin-layer electrophoresis, after hydrolyzing the *N*-acetylaminoacyl-$tRNA^{Lys}$ species in alkaline solution, as described elsewhere (Wagner & Sprinzl, 1982). EF-Tu·GTP was prepared by incubation of a corresponding amount of EF-Tu·GDP (see legends to the figures for final concentrations) in TMAKD buffer containing 10 mM phosphoenolpyruvate, 200 μg/mL pyruvate kinase, and 1 mM GTP at 37 °C for 10 min. The mixture was kept on ice before its use but not longer than 1 h.

Results

Different ^{14}C-labeled *N*-acetylaminoacyl-$tRNA^{Lys}$ analogues were bound to poly(A)-programmed 70S ribosomes. The ribosomal complexes were then treated with EF-G and GTP in order to translocate the peptidyl-tRNA analogues to the ribosomal P site. After the addition of native [^{3}H]Lys-$tRNA^{Lys}$-CpCpA·EF-Tu·GTP, the rate of incorporation of [^{3}H]lysine into polypeptides was measured. The results shown in Figure 1a demonstrate that the most efficient incorporation is achieved if the polypeptide synthesis is initiated with the misaminoacylated Ac-Phe-$tRNA^{Lys}$-CpCpA. Ribosome-bound peptidyl-tRNAs derived from "deoxy"-tRNA species markedly inhibit the polymerization process. The extent of inhibition is independent of the point of attachment of the acetylaminoacyl residues to the tRNA (2′- or 3′-hydroxyl) and of the nature of the amino acid (phenylalanine or lysine). If the radioactivity originating from the incorporation of ^{14}C-labeled *N*-acetyl amino acids into the growing polypeptide is measured (Figure 1b), the presence of the N-terminal *N*-acetyl amino

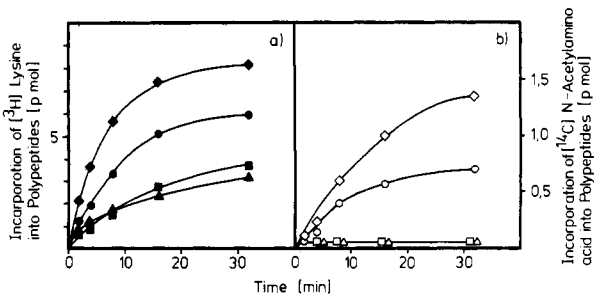

FIGURE 1: Initiation of polylysine synthesis on poly(A)-programmed posttranslocational ribosomes. 47 A_{260} units/mL 70S ribosomes were incubated with 1.1 mg/mL poly(A) and 1.1 μM *N*-acetyl[^{14}C]-aminoacyl-$tRNA^{Lys}$ species (specific activity 342 Ci/mol for lysine and 513 Ci/mol for phenylalanine) in 45 μL of TMAKD buffer for 20 min at 37 °C. After the addition of EF-G and GTP (final concentrations 500 μg/mL and 1 mM, respectively), the incubation of the reaction solutions (now 50 μL) was continued for 10 min. Then 22 μL of a TMAKD solution containing 4.7 μM [^{3}H]Lys-$tRNA^{Lys}$-CpCpA (specific activity 2.5 Ci/mmol) and 28 μM EF-Tu·GTP was added and the mixture was again incubated at 37 °C. Aliquots of 12 μL were withdrawn at time intervals during 32 min and analyzed for the formation of polylysine according to Gardner et al. (1962). The incorporation of [^{3}H]lysine (a) and the N-terminal *N*-acetyl[^{14}C]aminoacyl residues (b) into the polypeptides was determined. Polypeptide synthesis started with Ac_2-Lys-$tRNA^{Lys}$-CpCpA (●, ○), Ac-Phe-$tRNA^{Lys}$-CpCpA (◆, ◇), Ac_2-Lys-$tRNA^{Lys}$-CpCp2′dA (▲, △), and Ac-Phe-$tRNA^{Lys}$-CpCp3′dA (■, □).

acid in the polypeptide can only be detected in the case of the Ac_2-Lys-$tRNA^{Lys}$-CpCpA and Ac-Phe-$tRNA^{Lys}$-CpCpA, derived from native tRNA. Neither Ac-Phe-$tRNA^{Lys}$-CpCp3′dA nor Ac_2-Lys-$tRNA^{Lys}$-CpCp2′dA is able to transfer their *N*-[^{14}C]acetylaminoacyl residues to the acid-precipitable polymer. This result shows that those ribosomes occupied by the modified peptidyl-tRNA analogues are not competent in the elongation cycle. The slow incorporation of [^{3}H]lysine into polylysine observed in these experiments shown in Figure 1a therefore can be attributed either to 70S ribosomes in which the P site was vacant prior to addition of [^{3}H]Lys-$tRNA^{Lys}$-CpCpA or to an exchange between modified peptidyl-tRNA analogues and native [^{3}H]Lys-$tRNA^{Lys}$-CpCpA.

The inhibition of initiation observed in the case of Ac-Phe-$tRNA^{Lys}$-CpCp3′dA is plausible, since we have already shown (Wagner et al., 1982), that this species is not active as a donor of the peptidyl residue. However, with Ac_2-Lys-$tRNA^{Lys}$-CpCp2′dA which is active as a donor, some step succeeding the peptidyl-transfer reaction must be inhibited. As a possibility, we have considered that the ejection of the deacylated $tRNA^{Lys}$-CpCp2′dA after the peptidyl transfer is retarded. The experiment shown in Figure 2 supports this assumption. The $tRNA^{Lys}$-CpCp2′dA was radioactively labeled by the incorporation of 2′-deoxy[^{3}H]adenosine 5′-monophosphate into the 3′ terminus of $tRNA^{Lys}$-CpC. It was subsequently aminoacylated with [^{14}C]lysine and the lysyl residue was N-acetylated, yielding Ac_2-[^{14}C]Lys-$tRNA^{Lys}$-CpCp[^{3}H]2′dA. This was bound to poly(A)-programmed ribosomes in the presence of EF-G and GTP, and [^{14}C]Lys-$tRNA^{Lys}$·EF-Tu·GTP was subsequently added. After incubation, the ribosomal complexes formed were separated from excess tRNA and amino acid by gel filtration. The elution profile (Figure 2) shows that under the chosen conditions, which should allow polypeptide synthesis to proceed, a considerable amount of the ^{3}H radioactivity from Ac_2-[^{14}C]-Lys-$tRNA^{Lys}$-CpCp[^{3}H]2′dA remains bound to the ribosomes. The molar ratio of ^{14}C-labeled to ^{3}H-labeled substances eluted with the ribosomal complexes in the exclusion volume is about 2 to 1. When a similar control experiment was performed with [^{14}C]peptidyl-tRNA-CpCp[^{3}H]A derived from native

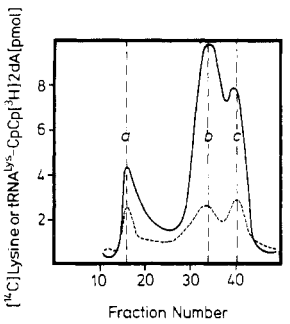

FIGURE 2: Gel filtration of poly(A)-programmed 70S ribosomes with bound Ac_2-[^{14}C]Lys-tRNA^{Lys}-CpCp[^{3}H]2′dA after incubation with [^{14}C]Lys-tRNA^{Lys}-CpCpA in the presence of elongation factors Tu and G. The reaction mixture contained 64 A_{260} units/mL 70S ribosomes, 1.7 mg/mL poly(A), and 1.7 μM Ac_2-[^{14}C]Lys-tRNA^{Lys}-CpCp[^{3}H]2′dA (specific activity of lysine 50 Ci/mol; for preparation see Materials and Methods) in 25 μL of TMAKD buffer. After 20 min at 37 °C, 500 μg/mL EF-G and GTP (final concentration 1 mM) were added and the incubation was continued for 5 min. To 30 μL of this solution, 25 μL of a mixture containing 3 μM [^{14}C]Lys-tRNA^{Lys}-CpCpA (specific activity 50 Ci/mol) and 15 μM EF-Tu·GTP in TMAKD buffer was added at 0 °C. The mixture was further incubated at 37 °C for 10 min and then injected onto a Sepharose 6B column (0.8 × 19 cm) at 4 °C (Girbes et al., 1976). The column had been equilibrated with TMAKD buffer at 4 °C. The elution profile was monitored by determining the ^{3}H (---) and [^{14}C] (—) radioactivity in the collected fractions (0.17 mL). The elution volumes of polyribosomes (a), aminoacyl-tRNA (b), and amino acids (c) were determined by appropriate standards.

tRNA^{Lys}, the nascent ^{14}C-labeled peptidyl residue remains attached to the ribosomal complex and the corresponding radioactivity appeared in the exclusion volume of the column. The radioactivity associated with the CpCp[^{3}H]A end of the tRNA on the other hand was retarded and eluated exclusively with the volume corresponding to tRNA. These experiments demonstrate that the dissociation of tRNA^{Lys}-CpCp2′dA from the ribosomal complexes did not take place before the process of translocation, whereas the native tRNA^{Lys} dissociated as expected. The ratio of ^{3}H/^{14}C radioactivity in the fast-eluting peak in Figure 2 indicated essentially that Ac_2-[^{14}C]Lys-[^{14}C]Lys-tRNA^{Lys}-CpCpA remained bound to the A site and tRNA^{Lys}-CpCp[^{3}H]2′dA to the P site of the ribosomes.

The ability of peptidyl-tRNA^{Lys}-CpCp2′dA to translocate from the ribosomal acceptor to the ribosomal donor site was investigated in the experiment shown in Figure 3. Here ^{3}H-labeled Ac-Phe-tRNA^{Lys}-CpCpA was bound to the poly(A)-programmed ribosomes in the presence of EF-G and GTP. To this complex [^{14}C]Lys-tRNA^{Lys}-CpCp2′dA and EF-Tu·GTP were added. After incubation, followed by alkaline hydrolysis of the reaction products, peptide analysis was performed by thin-layer electrophoresis. In contrast to the electropheretogram of acetyl[^{3}H]phenylalanine and [^{14}C]-lysine, given by the vertical broken lines in Figure 3, only one prominent new peak could be identified in Figure 3A. This occurred 4 cm to the anode side of the origin and must correspond to the dipeptide Ac-[^{3}H]Phe-[^{14}C]Lys as deduced from the 1:1 stoichiometry calculated from the ratio of ^{14}C to ^{3}H. In this experiment no polymerization of [^{14}C]lysine took place, since the prevailing part of the ^{14}C radioactivity appeared in the position corresponding to the [^{14}C]lysine standard. In Figure 3B the corresponding control experiment is shown. As in the experiment described above, Ac-[^{3}H]-Phe-tRNA^{Lys}-CpCpA was bound to the P site of the ribosomes, EF-G and GTP being present in the reaction mixture. Thus native [^{14}C]Lys-tRNA^{Lys} and EF-Tu·GTP were added to allow polymerization. Several new oligopeptides were synthesized. In this case the ^{14}C radioactivity migrated faster than the

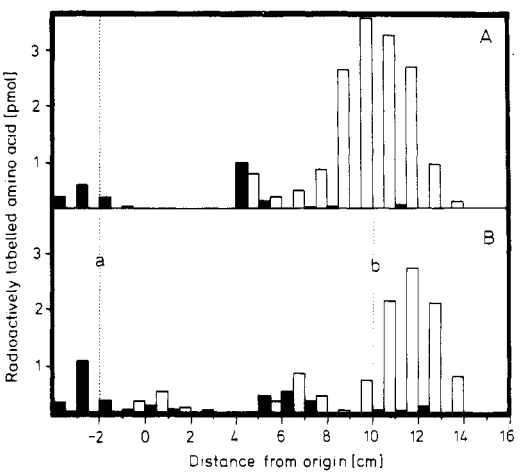

FIGURE 3: Thin-layer electrophoresis of the products formed on poly(A)-programmed 70S ribosomes with bound Ac-[^{3}H]Phe-tRNA^{Lys}-CpCpA and [^{14}C]Lys-tRNA^{Lys}-CpCp2′dA (A) or [^{14}C]-Lys-tRNA^{Lys}-CpCpA (B) in the presence of elongation factors G and Tu. 60 A_{260} units/mL 70S ribosomes were incubated with 1.5 mg/mL poly(A) and 2 μM Ac-[^{3}H]Phe-tRNA^{Lys}-CpCpA (specific activity 4 Ci/mmol) in 20 μL of TMAKD buffer at 37 °C for 15 min. GTP and EF-G to final concentrations of 1 mM and 1 mg/mL, respectively, were added (final volume 23 μL), and the incubation was continued at 37 °C for 5 min. 10 μL of this reaction solution was then mixed with 6 μL of 35 μM EF-Tu·GTP and (A) 10 μL of 3.3 μM [^{14}C]-Lys-tRNA^{Lys}-CpCp2′dA or (B) 10 μL of 3.2 μM [^{14}C]Lys-tRNA^{Lys}-CpCpA (specific activity of lysine 342 Ci/mol). Each of these mixtures was further incubated at 37 °C for 15 min. After the addition of 3 μL of 50% trichloroacetic acid the samples were kept at 0 °C for 10 min and the precipitates were collected by low-speed centrifugation thereafter. The supernatants were withdrawn and the pellets were hydrolyzed in 6 μL of 0.3 N NaOH at 37 °C for 15 min. The samples were neutralized by adding 3 μL of 1 N acetic acid and then applied onto a cellulose thin-layer sheet (20 × 20 cm). Electrophoresis was performed with 0.1 M ammonium acetate, pH 4.5, applying 380 V for 75 min. The sheet was dried, and where the samples had been applied, lanes of 2-cm width were cut out. These were sliced into 1 cm wide stripes. ^{3}H (closed bars) and ^{14}C radioactivity (open bars) on the stripes was measured. The positions where *N*-acetylphenylalanine (a) and lysine (b) migrate in the electropheretogram were determined by appropriate standards.

[^{14}C]lysine standard, indicating that extensive polymerization did occur. The peak of Ac-[^{3}H]Phe-[^{14}C]Lys appearing 5 cm toward the anode in Figure 3B is considerably smaller than the one in Figure 3A. As expected the ratio of ^{3}H:^{14}C gradually decreases with the growing peptide chain.

From the results of Figure 3 we conclude that in the case of the modified 3′-deoxy-tRNA species formation of the dipeptide takes place but that the translocation of Ac-[^{3}H]-Phe-[^{14}C]Lys-tRNA^{Lys}-CpCp2′dA from the A to the P site is blocked. If the above peptidyl-tRNA species had translocated to the ribosomal donor site and if only the release of tRNA^{Lys}-CpCp2′dA were inhibited, the formation of a tripeptide with a corresponding 2:1 ^{14}C:^{3}H molar ratio would have been expected as a major product. As seen in Figure 3A this is not the case.

Discussion

These experiments with nonisomerizable modified aminoacyl- and peptidyl-tRNAs point to a striking role of the 2′-hydroxyl group on the 3′-terminal adenosine of tRNA during EF-G-mediated translocation on the ribosome. In this not yet fully understood process, several changes in the topological arrangement of the substrates and the ribosomes must occur. After the peptidyl-transfer reaction the free tRNA is ejected from the ribosomal P site and, probably simultaneously, the peptidyl-tRNA·mRNA complex moves from the puromycin-

Table I: Activity of Aminoacyl-tRNA-CpCp2′dA and Peptidyl-tRNA-CpCp2′dA in Partial Reactions of Peptide Elongation

reaction	occupancy after the reaction: A site	P site	conclusion
		Experiment 1	
binding	Lys-tRNA^{Lys}-CpCpA	Ac_2-Lys-tRNA-CpCp2′dA	
peptidyl transfer	Ac_2-Lys-Lys-tRNA^{Lys}-CpCpA	tRNA^{Lys}-CpCp2′dA	peptidyl transfer takes place
translocation	unchanged	unchanged	tRNA^{Lys}-CpCp2′dA is not released
		Experiment 2	
binding	Lys-tRNA-CpCp2′dA	Ac-Phe-tRNA^{Lys}-CpCpA	
peptidyl transfer	Ac-Phe-Lys-tRNA^{Lys}-CpCp2′dA	tRNA^{Lys}-CpCpA	peptidyl transfer takes place
translocation	unchanged	unchanged	peptidyl-tRNA^{Lys}-CpCp2′dA is not transferred from A to P site

unsensitive A site to the puromycin-sensitive P site of the ribosome (Brot, 1977). The precise function of EF-G in this particular step is not clear. It was shown that the binding of EF-G and GTP to 70S ribosomes is required in order to dissociate the deacylated tRNA from the ribosomal P site (Lucas-Lenard & Haenni, 1969; Ishitsuka et al., 1970). Then the translocation of the peptidyl-tRNA·mRNA complex is expected to proceed spontaneously (Leder, 1973). This model, based on a simple substrate–product relationship between the peptidyl-tRNA and the respective ribosomal binding sites, is strongly supported by the recent determination of the corresponding binding parameters of peptidyl-tRNA to ribosomes (Holschuh & Gassen, 1982). Furthermore, the question about the number of the tRNA binding sites on the ribosomes is not settled. Whereas the generally accepted two binding site model was compatible for a long time with most of the experimental evidence, very recently a model suggesting the simultaneous binding of three tRNA molecules to the ribosomes has been presented (Rheinberger et al., 1981).

The binding of EF-G and GTP to 70S ribosomes and the following hydrolysis of GTP to GDP are not coupled to the translocation process, since it also takes place in the absence of tRNAs and mRNA (Brot, 1977). A direct interaction of A site bound peptidyl-tRNA or P site bound deacylated tRNA with EF-G is therefore not anticipated. The results of our experiments, which are summarized in Table I, suggest that the release of deacylated tRNA and the translocation of the peptidyl-tRNA·mRNA complex are not spontaneous. The absence of 2′-hydroxyl group on the 3′-terminal adenosine of tRNA completely blocks translocation. Even in the presence of EF-G and GTP neither the peptidyl-tRNA-CpCp2′dA·mRNA complex nor the deacylated tRNA-CpCp2′dA can move, thus locking the ribosomes in their pretranslocation state. It could not be decided from these experiment, which of the EF-G-catalyzed reactions is blocked by the tRNA^{Lys}-CpCp2′dA species.

Up to now two well-defined inhibitors of translocation have been described. Fusidic acid blocks the dissociation of the EF-G·GDP complex from ribosomes. In this case peptide bond formation takes place and the peptidyl-tRNA translocates from the A to the P site of the ribosomes. The binding of the next aminoacyl-tRNA·EF-Tu·GTP ternary complex to the ribosomal A site is, however, inhibited, probably due to the common EF-Tu and EF-G ribosomal binding sites. Thus, the inhibition by fusidic acid leads to the completion of one elongation cycle and locks the ribosomes in their posttranslocational state (Inoue-Yokosawa et al., 1973).

The replacement of GTP by its nonhydrolyzable analogue GMPPCP leads to an effect similar to that of the fusidic acid inhibition, since the hydrolysis of GTP to GDP is probably required for the dissociation of EF-G as the EF-G·GDP complex. Again one elongation cycle takes place, but the binding of the next aminoacyl-tRNA·EF-Tu·GTP ternary complex is inhibited by the presence of EF-G·GMPPCP on the ribosomes. In the presence of the nonhydrolyzable analogue of GTP, the ribosomes also remain in their posttranslocational state (Inoue-Yokosawa et al., 1973).

If aminoacyl-tRNA^{Lys}-CpCp2′dA is bound to the ribosomal A site, peptide bond formation occurs but the translocation of the formed peptidyl-tRNA^{Lys}-CpCp2′dA to the P site is inhibited. Similarly the release of the deacylated tRNA from the ribosomal P site is dependent on the presence of a free vicinal cis diol function on the 3′ end of the tRNA. From this we conclude that the recognition of an intact 3′ end of both peptidyl-tRNA and deacylated tRNA by some ribosomal components, or by EF-G, is required before translocation can proceed. By use of aminoacyl-tRNA-CpCp2′dA for the first time a method is described that enables to lock ribosomal complexes in their pretranslocational stage even in the presence of EF-G and GTP.

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Separation and Comparison of 2-Thioribothymidine-Containing Transfer Ribonucleic Acid and the Ribothymidine-Containing Counterpart from Cells of *Thermus thermophilus* HB 8†

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ABSTRACT: For the extreme thermophile *Thermus thermophilus* HB 8, a positive correlation was observed among the growth temperatures of the cells, the melting temperature, and the 2-thioribothymidine (s^2T) content of tRNA extracted from cells grown at various temperatures [Watanabe, K., Shinma, M., Oshima, T., & Nishimura, S. (1976) *Biochem. Biophys. Res. Commun. 72*, 1137-1144]. On the basis of these observations, studies were carried out from which the following results were obtained. (1) Both RNase T_1 and U_2 digestions of tRNA gave only four fragments containing s^2T or T: $s^2T\psi CGp$, $s^2T\psi CAp$, $T\psi CGp$, and $T\psi CAp$. For the different growth temperatures, the ratio of the content of $s^2T\psi CGp$ plus $s^2T\psi CAp$ to that of $T\psi CGp$ plus $T\psi CAp$ was almost the same as that of the s^2Tp to Tp content reported previously. (2) The midpoint of the s^2T-specific circular dichroism spectral change induced by heat was constant for all tRNAs extracted from cells grown at various temperatures, suggesting that the s^2T-containing tRNAs melt at about the same temperature, which is independent of the growth temperature of cells. (3) s^2T-containing tRNA was separated from the T-containing counterpart quantitatively by a specific modification of s^2T with bromoaceto-2,4-dinitroanilide followed by BD-cellulose column chromatography. The molar ratio of the s^2T- and T-containing tRNAs was also similar to that of s^2Tp to Tp as mentioned above. These results demonstrate that *T. thermophilus* cells have both s^2T- and T-containing tRNAs, whose relative content is controlled by the growth temperature. This phenomenon may be necessary to enable the thermophile to adapt to higher temperatures.

An extreme thermophile, *Thermus thermophilus* HB 8, is capable of growing over a wide temperature range between 48 and 85 °C (Oshima & Imahori, 1974). The melting temperatures of unfractionated tRNAs from cells cultured at various temperatures (50, 55, 60, 68, 75, and 80 °C) showed strong correlations to the different culture medium temperatures. In addition, the 2-thioribothymidine (s^2T)[1] content of the tRNAs was also proportional to the growth temperature of the cells (Watanabe et al., 1976a).

These results imply the following possibilities: (1) the thermostability of the thermophile tRNA is mainly determined by the content of s^2T; (2) in the cells of *T. thermophilus* HB 8, two species of tRNA exist, one s^2T-containing tRNA and another ribothymidine- (T) containing tRNA. The ratio of these two species is determined by the growth temperatures of the cells.

The first possibility was confirmed by CD (Watanabe et al., 1976b) and NMR studies (Davanloo et al., 1979) and other biochemical approaches (Watanabe et al., 1980; Kumagai et al., 1982); however, there has been no evidence for the second possibility.

In this paper, we try to refine the previous observations by confirming the following points: First, the s^2T residue in fact replaces only the T residue in the $T\psi C$ sequence, by analysis of all the fragments containing T or s^2T, quantitatively. Second, we present evidence for the existence of the two tRNA species in *T. thermophilus* cells, by direct separation of s^2T-containing tRNA and its T-containing counterpart using a specific chemical modification on the s^2T residue. This process was performed by synthesizing a reagent that modifies thio-

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[1] Abbreviations: s^2T, 2-thioribothymidine; BADA, bromoaceto-2,4-dinitroanilide; C=S, thiocarbonyl group; CDTA, *trans*-1,2-diaminocyclohexanetetraacetic acid; H_2O_2, hydrogen peroxide; LC, high-performance liquid chromatography; DCC, *N,N'*-dicyclohexylcarbodiimide; Me_2SO, dimethyl sulfoxide; Me_4Si, tetramethylsilane; CD, circular dichroism; NMR, nuclear magnetic resonance; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; BD, benzoylated diethylaminoethyl.

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