N^{ϵ}-Acetyllysine Transfer Ribonucleic Acid: A Biologically Active Analogue of Aminoacyl Transfer Ribonucleic Acids†

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ABSTRACT: Unfractionated *Escherichia coli* tRNA has been aminoacylated with lysine and preferentially acetylated at the ϵ-amino nitrogen of lysine by reaction with *N*-acetoxysuccinimide. After treatment with peptidyl-tRNA hydrolase, 90% of the aminoacylated tRNA molecules were N^{ϵ}-acetyl-Lys-tRNA. Post-ribosomal supernatant enzymes would not deacylate N^{ϵ}-acetyl-Lys-tRNA in the presence of AMP and PP_i, even though such mixed enzymes could acylate, with lysine, tRNA which had been exposed to the acetylation reaction conditions. Poly(rA) stimulated the binding of N^{ϵ}-acetyl-Lys-tRNA to *E. coli* ribosomes. At the ribosome and tRNA concentrations used, N^{ϵ}-acetyl-Lys-tRNA was bound nearly as well as Lys-tRNA at 30 mM Mg^{2+}; at 10 mM Mg^{2+}, the analogue was bound one-half as well as Lys-tRNA. Both Lys-tRNA and N^{ϵ}-acetyl-Lys-tRNA reacted only slightly with puromycin at either 10 or 30 mM Mg^{2+}. When Lys-$tRNA_{E.\ coli}$ or N^{ϵ}-acetyl-Lys-$tRNA_{E.\ coli}$ were added to rabbit reticulocyte cell-free protein synthesizing incubations, the incorporation of either amino acid into protein was complete within 5 min. The final incorporation level of the analogue was 82% that of the unmodified lysine. After protein synthesized in the presence of N^{ϵ}-acetyl-[^{14}C]Lys-tRNA had been digested enzymatically to single amino acids, ion-exchange chromatography and paper electrophoresis showed that nearly all of the radioactivity was present as N^{ϵ}-acetyllysine. Gel filtration of the post-ribosomal supernatant revealed that most of the N^{ϵ}-acetyllysine radioactivity cochromatographed with tetrameric hemoglobin.

Analogues of aminoacyl-tRNA provide a means of investigating the structural relations of tRNA and ribosomes throughout the elongation cycle of protein biosynthesis. In particular, such analogues make possible studies of elongation factor T-dependent ribosomal functions and conformations. Investigations utilizing aminoacyl-tRNA analogues would also complement the recent photoaffinity and chemical affinity labeling studies of ribosomal topography which have employed analogues of peptidyl-tRNA (see, for example, Czernilofsky et al., 1974; Eilat et al., 1974; Girshovich et al., 1974; Hauptmann et al., 1974; Hsiung et al., 1974; Pellegrini et al., 1974; Sopori et al., 1974, and references therein).

To date, two aminoacyl-tRNA analogues have been prepared, one by a modification of Val-tRNA at the 4-thiouridine, located at the joint between the amino acid accepting stem and the dihydrouridine loop (Schwartz and Ofengand, 1974), and one by the addition of a spin-label to the amino acid side chain of Cys-tRNA (Kabat et al., 1970). No report has appeared on the biological activity of the latter. We now report a method for preparing, from unfractionated tRNA, analogues of Lys-tRNA in which a substituent is covalently attached to the ϵ-amino group of the lysine side chain. These analogues can be used to probe the functional sites of the ribosome near the peptide transferase center; they offer the additional possibility of transferring the modified amino acid, with probe, into the nascent protein chain. N^{ϵ}-Acetyl-Lys-tRNA (ϵ-Ac-Lys-tRNA),[1] prepared by our procedures, retains a high level of biological activity, only slightly less than that of unmodified Lys-tRNA. In particular, N^{ϵ}-acetyllysine is transferred from ϵ-Ac-Lys-$tRNA_{E.\ coli}$ into hemoglobin in a rabbit reticulocyte cell-free protein synthesizing system.

Experimental Section

Ribosomes, Supernatant Enzymes, and Peptidyl-tRNA Hydrolase. E. coli K-12, strain CA 244 (Hfr, λ^+, tryp$^-$, lac_z^-, $T1^R$), were grown to late exponential phase, harvested, and frozen as described by Menninger et al. (1970). The S-100 fraction was separated into partially purified peptidyl-tRNA hydrolase and S-100 enzymes by chromatography on DEAE-cellulose as described earlier (Menninger et al., 1970). Ribosomes were washed three times in 1 M NH_4Cl; residual peptidyl-tRNA was removed by incubation with 1 mM puromycin, as described by Harris and Pestka (1973).

Lys-tRNA. Unfractionated *E. coli* K-12 tRNA (General Biochemicals) was aminoacylated with [^{14}C]lysine at 37°C for 30 min in 12-ml incubations as described by Menninger et al. (1970). The Lys-tRNA was separated from protein by chromatography at 4°C on a BD-cellulose column (1.8 cm

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[1] Abbreviations used are: ϵ-Ac-Lys-tRNA, N^{ϵ}-acetyl-Lys-tRNA; α-Ac-Lys-tRNA, N^{α}-acetyl-Lys-tRNA; DiAc-Lys-tRNA, *N,N*-diacetyl-Lys-tRNA; BD-cellulose, benzoylated DEAE-cellulose, prepared by the method of Gillam et al. (1967); CFS I, cell-free system I (Woodward et al., 1974a); 1 A_{260} unit is the amount of material in 1.0 ml which would yield a value of 1.0 for the absorbance measured at 260 nm in a cuvette with a path length of 1.0 cm.

diam. × 2.8 cm) equilibrated with buffer I (0.3 M NaCl–10 mM sodium acetate (pH 4.5)–10 mM $MgSO_4$–1 mM $Na_2S_2O_3$). After the column was washed with buffer I, [^{14}C]Lys-tRNA, as well as most unacylated tRNAs, was eluted with buffer I made 1.5 M in NaCl and 10% (v/v) in ethanol. The tRNA was precipitated with ethanol, then resuspended in and dialyzed against buffer II (1 mM potassium acetate, pH 5.0).

ε-Ac-Lys-tRNA. The *N*-hydroxysuccinimide ester of acetic acid (*N*-acetoxysuccinimide) was prepared by the method of Lapidot et al. (1967), except that it was recrystallized out of 2-propanol instead of water. *N*-Acetoxysuccinimide (6.1–6.5 mg) in 0.5 ml of tetrahydrofuran was added to a 2.0-ml aqueous solution in ice containing 50 mM potassium phosphate (pH 7.0), 0.5 mM $Na_2S_2O_3$, and 256–275 A_{260} units of [^{14}C]Lys-tRNA. The apparent[2] pH was raised quickly with stirring to 10.8 with 2 N KOH. After 15 s at this high pH, the reaction was stopped by lowering the pH to less than 5 with 4 N acetic acid. Slow electrode response to the last addition of base makes precise pH adjustment difficult on this time scale; an apparent pH between 10.7 and 11.1 is acceptable for the 15-s reaction. On completion of the reaction, 2.5 ml of 2 M potassium acetate (pH 5.0) and then 15 ml of cold 95% ethanol were added to the reaction mixture, and the tRNA was precipitated overnight at −20°C. The tRNA was separated from residual reaction mixture components on a BD-cellulose column, as described for Lys-tRNA above, and resuspended in buffer II. Despite the exposure to high pH, the recovery of specific activity (picomoles of Lys/A_{260} unit of tRNA), assayed by cold trichloroacetic acid precipitation, was greater than 80%. To remove lysines which are acetylated at the α-amino nitrogen, the above tRNA solution was dialyzed against buffer II at 4°C and then incubated with peptidyl-tRNA hydrolase. These incubations were carried out at 30°C for 10 min in 2.4-ml volumes containing 100 mM ammonium cacodylate (pH 7.3), 5 mM $MgCl_2$, 235–280 A_{260} units of acetylated Lys-tRNA, and 0.6 ml of partially purified peptidyl-tRNA hydrolase (Menninger et al., 1970). The ε-Ac-Lys-tRNA was recovered from the incubation by BD-cellulose chromatography as described for Lys-tRNA above.

Electrophoresis. To analyze the degree of acetylation of the lysine, an aliquot of the acetylated Lys-tRNA solution was mixed with an equal volume of 0.1 M triethylamine and incubated at 37°C for 60–90 min to hydrolyze the aminoacyl bond. This solution was dried in a stream of N_2, resuspended in water, spotted on Whatman 3MM paper, and electrophoresed at either pH 1.9 (105 min, 42 V/cm) or at pH 3.6 (40 min, 55 V/cm). After drying, the strips were cut into 1-cm segments and counted in toluene based scintillator (Menninger et al., 1970). Lysine, N^{ϵ}-acetyllysine (Cyclo Chemical), and N^{α}-acetyllysine (Sigma) markers were located using a ninhydrin stain.

Ribosome Binding. The assay procedures were similar to those of Nirenberg and Leder (1964), except that ribosomes and poly(rA) were preincubated at 37°C for 25 min before combination with aminoacyl-tRNA and salts. The 20°C, 30-min final incubations (50 μl) contained 100 mM Tris-acetate (pH 7.2), 50 mM NH_4Cl, $MgCl_2$ as indicated in Figure 2, 2 A_{260} units of ribosomes, and either 0.15 A_{260} unit of [^{14}C]Lys-tRNA (31 pmol of Lys/A_{260} unit of tRNA; 310 mCi/mmol of Lys) or 0.15 A_{260} unit of ε-Ac-[^{14}C]Lys-tRNA (32 pmol of Lys/A_{260} unit of tRNA; 310 mCi/mmol of Lys); the "+ poly(rA)" incubations also contained 0.31 A_{260} unit of poly(rA). The particular ε-Ac-Lys-tRNA solution used in these and in the puromycin reactivity assays was 92% ε-Ac-Lys-tRNA, 6% diAc-Lys-tRNA, 0.4% α-Ac-Lys-tRNA, and 1.6% Lys-tRNA; roughly one-half of the $tRNA^{Lys}$ in the solution is unacylated.

Puromycin Reactivity. Puromycin reactivity assays differed from the ribosome binding assays only in the following respects: all incubations contained 0.31 A_{260} unit of poly(rA); some final incubations contained 0.48 mM puromycin (dihydrochloride, pH 7.0); and final incubations (30 min, 37°C) were assayed by cold trichloroacetic acid precipitation. The difference in the cold trichloroacetic acid precipitable radioactivity between the averages of duplicate incubations with and without puromycin indicated the puromycin-reacted radioactivity.

N^{ϵ}-Acetyllysylhemoglobin. Rabbit reticulocyte CFS I lysates, hemin, and master mix were prepared as described by Woodward et al. (1974a). The ε-Ac-[^{14}C]Lys-tRNA preparation used in the reticulocyte experiments had the following characteristics: 29 pmol of Lys/A_{260} unit of tRNA; 310 mCi/mmol of Lys; 126 A_{260} units/ml; 92% ε-Ac-Lys-tRNA, 6% diAc-Lys-tRNA, 0.4% α-Ac-Lys-tRNA, and 1.6% Lys-tRNA. CFS I lysate (2.4 ml), 300 μl of master mix (without radioactive leucine), and 25 μl of hemin were combined and gently stirred. Two milliliters of this lysate–master mix–hemin solution was then combined with 175 μl of ε-Ac-Lys-tRNA and 50 μl of water and incubated at 37°C with gentle shaking. After 60 min the solution was diluted with 4.0 ml of water and centrifuged at 105 000*g* for 180 min to remove the ribosomes. A 1.0-ml rinse of the pellet with 4 mM $MgCl_2$ was combined with the supernatant, and this post-ribosomal supernatant was stored at −20°C. Nearly all (97%) of the radioactivity added to the incubation was recovered; 97% of the recovered radioactivity was present in the post-ribosomal supernatant.

Enzymatic Digestion of N^{ϵ}-Acetyllysylhemoglobin and Ion-Exchange Chromatography. Heme was removed and the cysteine residues were aminoethylated as reported elsewhere (Woodward et al., 1974b). No attempt was made either to separate the α and β chains or to analyze tryptic peptides since both methodologies require lysines with unblocked ε-amino groups (Dintzis, 1961; Woodward et al., 1974b). An aqueous solution containing about 20 mg of the aminoethylated N^{ϵ}-acetyllysylglobin was brought to 0.08 M NH_4HCO_3 (pH 8.1). Trypsin (0.25 mg; Worthington) and α-chymotrypsin (0.25 mg; Worthington), freshly dissolved in 1 mM HCl, were added and the solution was incubated with stirring at 37°C; fresh trypsin (0.25 mg) and chymotrypsin (0.25 mg) were added after 8 h. After 24 h, the pH of the solution was lowered to 7.2 and the α-chymotrypsin was irreversibly inactivated by incubation with 1.4 mM TPCK (1.35 mg of L-1-tosylamido-2-phenylethyl chloromethyl ketone (Sigma) in 100 μl of methanol) at room temperature for 60 min with stirring (Schoellmann and Shaw, 1963). This avoids possible digestion of the aminopeptidase by α-chymotrypsin; trypsin does not inactivate aminopeptidase M (DeLange and Smith, 1971). After raising the pH back to 8.0, 2000 mU of aminopeptidase M (Henley and

[2] We use "apparent pH" to signify the pH measured by a Model 12 Corning pH meter and a 476050 Corning electrode in this mixed solvent (20% tetrahydrofuran and 80% aqueous) solution. The addition of the tetrahydrofuran to the pH 7.0 aqueous buffer increased the measured pH to 7.5–7.8 without any addition of base. Hence the requirement for the term "apparent pH".

Co.) in water was added to the digestion mixture. The mixture was then incubated for a total of 24 h at 37°C with stirring; fresh aminopeptidase M (2000 mU) was added to the mixture at 6.5 h. For chromatography the pH of the digestion solution was reduced to less than 2 with 6 N HCl. After adding 2×10^5 dpm of [^{3}H]lysine as a marker, the digest was chromatographed at 50°C on a Dowex 50 column as described elsewhere (Woodward et al., 1974b).

Results

Preparation of ϵ-Ac-Lys-tRNA. The reaction conditions necessary to acetylate preferentially the lysine ϵ-amino nitrogen of Lys-tRNA are described in the Experimental Section. The apparent pH must be sufficiently high to deprotonate the ϵ-NH_2 group of the lysine, which has an aqueous pK_a near 10.5 (Greenstein and Winitz, 1961). An apparent pH of 10.4–10.5 was not high enough to allow complete reaction of the ϵ-NH_2 with the *N*-acetoxysuccinimide during the 15-s reaction period. On the other hand, an apparent pH of 12–13 during the reaction was too high because the deacylation of the aminoacyl-tRNA was substantial, much reducing the yield of ϵ-Ac-Lys-tRNA. The optimal apparent pH of 10.8–11.0 for the 15-s reaction allowed the complete (98%) reaction of the ϵ-NH_2 with *N*-acetoxysuccinimide while minimizing the deacylation.

Ten seconds at high pH was not sufficient for the complete reaction of the ϵ-NH_2 with *N*-acetoxysuccinimide. Exposure to high pH for more than 15 s lowered the yield of ϵ-Ac-Lys-tRNA by allowing time for additional acetylation of the α-NH_2 and deacylation of the aminoacyl-tRNA. It is important to increase the apparent pH to 10.8–11.0 as quickly as possible, in order to minimize the time available for α-NH_2 acetylation. Raising the pH slowly resulted in a much larger proportion of diAc-Lys-tRNA product.

N-Acetoxysuccinimide reacts with both the α- and ϵ-amino groups of lysine. To determine the relative amounts of the four reaction products, the radioactive lysine species were hydrolyzed from the tRNA and subjected to electrophoresis. Radioactive lysine, N^{α}-acetyllysine, and N^{ϵ}-acetyllysine were identified by coelectrophoresis with markers. The fourth major component was presumed to be *N,N*-diacetyllysine. The following observations support this assignment: the putative diAc-lys-tRNA, compared to Lys-tRNA, was much more susceptible to the action of peptidyl-tRNA hydrolase (see below), and less susceptible to chemical deacylation, both in the presence and in the absence of Cu^{2+} ions (A. E. Johnson and J. R. Menninger, unpublished data). Also, the electrophoretic mobility of the fourth peak was appropriate to our identification. Various reactions have yielded products which were 68–77% ϵ-Ac-Lys-tRNA, 20–30% diAc-Lys-tRNA, 0.1–0.7% α-Ac-Lys-tRNA, and 0.7–1.0% Lys-tRNA.

Since *N*-acetoxysuccinimide reacts with unprotonated amines, one would expect the extent of reaction to be greater with the α-amino groups (pK_a about 7.5) than with the ϵ-amino groups (pK_a about 10.5) (Greenstein and Winitz, 1961). The fact that ϵ-acetylation is much faster probably means that the partially unprotonated ϵ-NH_2 groups are much more accessible for reaction. This is also evident in longer reactions (5–40 min) at either pH 5 or 6, in which the total amount of ϵ-acetylation is about the same as the total amount of α-acetylation (A. E. Johnson and J. R. Menninger, unpublished data). Other investigators have assumed that the primary nucleophile in a reaction requiring an unprotonated amine is an α-amino group if the pH is below 9.0 (Huang and Cantor, 1972). However, in our reactions the ϵ-amino groups constitute a much greater proportion of the reactive nucleophiles than anticipated on the basis of pH only. It is clear that the relative reactivities of macromolecule-associated α- and ϵ-amino groups are markedly affected by the solvent and/or steric environments immediately surrounding the putative nucleophiles.

To reduce the amount of N^{α}-acetylated products, the solutions were incubated with partially purified peptidyl-tRNA hydrolase from *E. coli*. This enzyme catalyzes the hydrolysis of the aminoacyl ester bond of any N^{α}-substituted aminoacyl-tRNA, except for N^{α}-substituted Met-$tRNA^{fMet}$ (Chapeville et al., 1969; Menninger et al., 1970; Kössel, 1970). A 10-min, 30°C incubation with peptidyl-tRNA hydrolase, as described in the Experimental Section, removed 76–84% of the N^{α}-acetylated lysines from their tRNAs. Neither a longer incubation (15 min) nor a second incubation with peptidyl-tRNA hydrolase removed the rest of the *N,N*-diacetyllysines. Chapeville et al. (1969) have also observed N^{α}-substituted aminoacyl-tRNA molecules which were insensitive to the action of peptidyl-tRNA hydrolase. Further incubation at pH 7.3 and 30°C does, however, reduce the amount of the desired ϵ-Ac-Lys-tRNA by chemical hydrolysis of the aminoacyl ester bond. Thus, a single 10-min, 30°C incubation with peptidyl-tRNA hydrolase maximizes the yield of ϵ-Ac-Lys-tRNA while minimizing the yield of diAc-Lys-tRNA. Various preparations, after peptidyl-tRNA hydrolase treatment, contained 87–93% ϵ-Ac-Lys-tRNA, 6–7% diAc-Lys-tRNA, 0.3–0.7% α-Ac-Lys-tRNA, and 0.9–1.6% Lys-tRNA.

Accepting Activity of tRNA Exposed to Acetylation Reaction Conditions. To examine the effect of the modification procedures on the biological activity of the tRNA, we tested the lysine accepting activity (Menninger, 1971) of various tRNAs. The unacylated, unfractionated tRNA control was charged to a specific activity of 69 pmol of Lys/A_{260} unit of tRNA. Exposing the tRNA to the acetylation reaction procedures decreased the level of aminoacylation by only 4%, to 66 pmol of Lys/A_{260} unit of tRNA. The reduction in accepting activity which did occur was found to result from the brief exposure to high pH and possibly reflects the base hydrolysis of a functionally important phosphodiester linkage. Aliquots of an ϵ-Ac-[^{14}C]Lys-tRNA solution (24 pmol of Lys/A_{260} unit of tRNA) were aminoacylated to a total specific activity of 67 pmol of Lys/A_{260} unit of tRNA with [^{3}H]lysine. Parallel aliquots, stripped of their N^{ϵ}-acetyllysine in 0.5 M Tris-Cl (pH 9.6) for 60 min at 37°C (to 0.9 pmol of Lys/A_{260} unit of tRNA), were charged to 65 pmol of Lys/A_{260} unit of tRNA. If any acetylation of the tRNA macromolecule occurred during the acetylation reaction, it did not interfere with the $tRNA^{Lys}$/lysyl-tRNA synthetase recognition and interaction.

Synthetase Recognition of ϵ-Ac-Lys-tRNA. According to the overall chemical equilibrium which describes the aminoacylation of (lysine) tRNA:

$$\text{Lys} + \text{ATP} + \text{tRNA}^{\text{Lys}} \overset{\text{lysyl-tRNA synthetase}}{\rightleftharpoons} \text{Lys-tRNA}^{\text{Lys}} + \text{AMP} + \text{PP}_i$$

supernatant enzymes, which include the lysyl-tRNA synthetase, should deacylate Lys-tRNA in the presence of AMP and PP_i. As shown in Figure 1, they did. This deacylation was AMP + PP_i dependent and not due to an RNase contaminant. However, under the same conditions very little enzymatic deacylation of ϵ-Ac-Lys-tRNA occurred

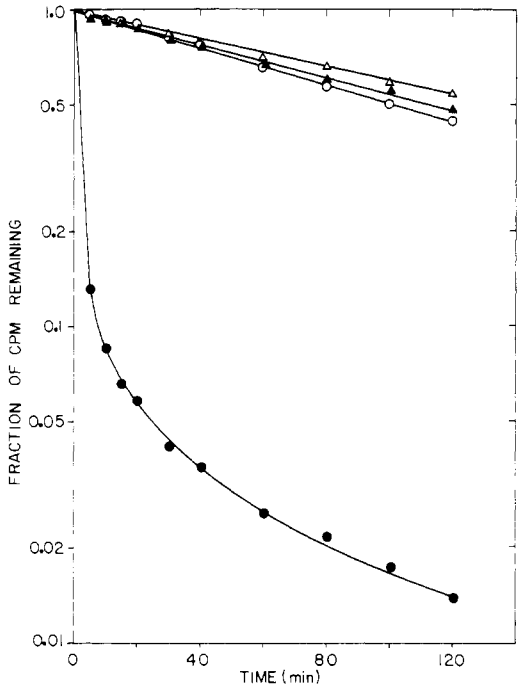

FIGURE 1: The enzymatic and nonenzymatic deacylation of Lys-tRNA and ε-Ac-Lys-tRNA. Incubations at 30°C contained, in 0.50 ml: 100 mM ammonium cacodylate (pH 7.2); 50 mM NH_4Cl; 6 mM $MgCl_2$; 4 mM AMP; 4 mM potassium pyrophosphate (pH 7.0); 7 mM mercaptoethanol; either 13.7 A_{260} units of [^{14}C]Lys-tRNA (43 pmol of Lys/A_{260} unit of tRNA; 72 mCi/mmol of Lys) (○, ●) or 11.9 A_{260} units of ε-Ac [^{14}C]Lys-tRNA (24 pmol of Lys/A_{260} unit of tRNA; 72 mCi/mmol of Lys; 91% ε-Ac-Lys-tRNA, 7% diAc-Lys-tRNA, 0.7% α-Ac-Lys-tRNA, and 1.5% Lys-tRNA) (△, ▲); either 0.10 ml of H_2O (○, △) or 0.10 ml of S-100 enzymes (●, ▲). The pH of the incubations at 30°C was 6.9. A 40-μl aliquot of a "- S-100" incubation or a 30-μl aliquot from a "+ S-100" incubation was removed from the incubations at the times indicated and assayed by cold trichloroacetic acid precipitation.

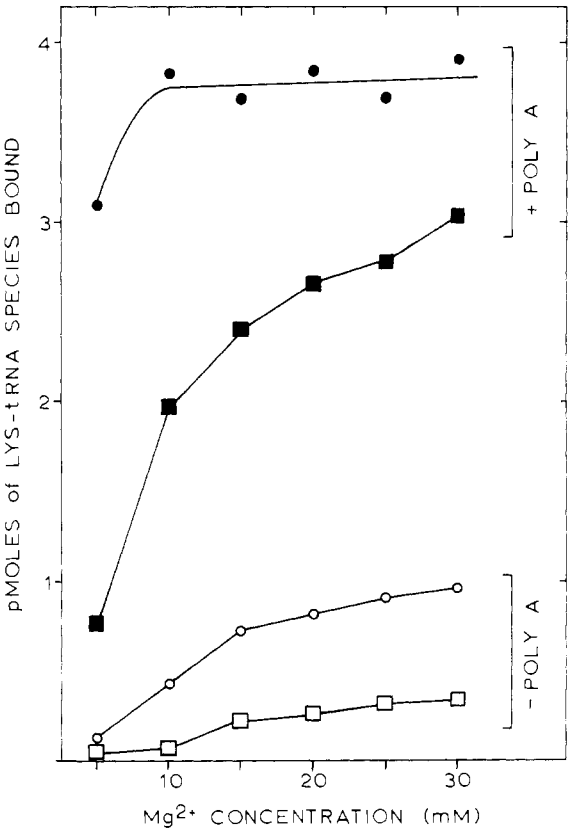

FIGURE 2: The magnesium ion concentration dependence of the binding of Lys-tRNA and ε-Ac-Lys-tRNA to ribosomes. Assays were performed as described in the Experimental Section. Filled symbols (●, ■) represent the binding to ribosomes in the presence of poly(rA); open symbols (○, □) represent the binding in the absence of poly(rA). Circles are data for Lys-tRNA (●, ○); squares for ε-Ac-Lys-tRNA (■, □).

(Figure 1). Since the tRNA portion of the molecule is capable of interacting with the synthetase (see above), the lack of synthetase deacylation must have been due to the acetylation of the ε-amino nitrogen. Thus, neither an ε-amide group nor an ε-hydroxyl (Stern and Mehler, 1965) group is a satisfactory replacement for the ε-amino group in lysyl-tRNA/synthetase recognition. It should be noted that the insensitivity of ε-Ac-Lys-tRNA to synthetase deacylation in the presence of AMP and PP_i provides a means of removing unlabeled Lys-tRNA from preparations of chemically modified Lys-tRNA.

Binding to Ribosomes. The binding affinities of ε-Ac-Lys-tRNA and Lys-tRNA to ribosomes were investigated in the absence of elongation factors. The magnesium ion dependence of this binding is shown in Figure 2. The stimulation of binding by poly(rA) is approximately the same for ε-Ac-Lys-tRNA and Lys-tRNA at 30 mM Mg^{2+}, though the total amount of ε-Ac-Lys-tRNA bound is less than that of Lys-tRNA. At lower Mg^{2+} concentrations the poly(rA)-stimulated binding of ε-Ac-Lys-tRNA is less than that of Lys-tRNA. The Lys-tRNA binding to ribosomes shows little Mg^{2+} dependence at this concentration of Lys-tRNA and ribosomes, since Lys-tRNA is limiting in the incubations. In the presence of added poly(U), slightly less Lys-tRNA or ε-Ac-Lys-tRNA bound to the ribosomes than in the absence of any added message. To ascertain that the ε-Ac-Lys-tRNA was not being deacetylated during an assay, the radioactivity bound to the ribosomes was extracted from a Millipore filter immediately after the binding assay washes and analyzed by paper electrophoresis. The single radioactive peak coincided with the N^{ϵ}-acetyllysine marker.

Puromycin Reactivity. Ribosome binding was measured at low aminoacyl-tRNA concentrations to maximize the site specificity of the tRNA binding. In order to determine the aminoacyl-tRNA binding site, puromycin reactivity was assayed under the same conditions as was the binding, except that the temperature of the final incubation was raised to 37°C: the binding of aminoacyl-tRNA to ribosomes was only slightly reduced at 37°C, but the rate of the puromycin reaction with oligo(Lys)-tRNA was much greater at 37 than at 20°C. Table I shows the puromycin reactivity of different tRNAs in the presence of ribosomes. Lys-tRNA and ε-Ac-Lys-tRNA apparently bound almost solely to the A site at either 10 or 30 mM Mg^{2+} at these aminoacyl-tRNA/ribosome concentrations. It is also possible that these aminoacyl-tRNAs do not react with puromycin when they are bound to the P site.

Incorporation of ε-Ac-[^{14}C]Lys-tRNA Radioactivity into Protein. The rabbit reticulocyte cell-free protein synthesizing system used in these experiments is almost as active as the intact cell for up to 20 min, and during a 60-min incubation the in vitro system is capable of synthesizing an average of 10–15 globin chains per ribosome (Woodward et al., 1974a). In preliminary experiments, performed as described by Woodward et al. (1974a,b), protein synthesis was measured as a function of the concentration of *E. coli*

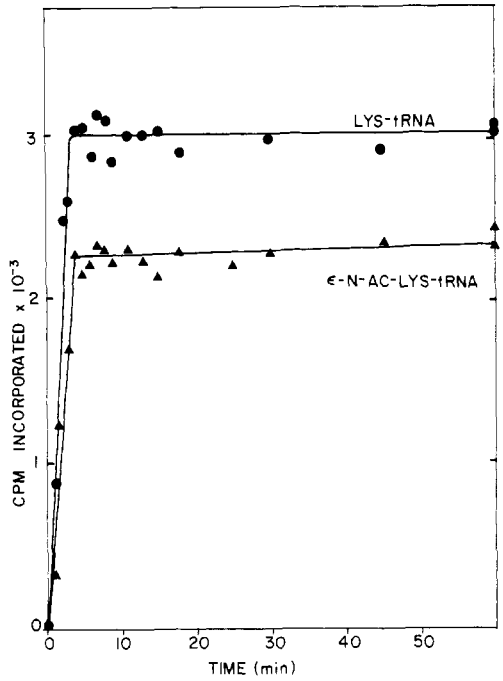

FIGURE 3: Incorporation of radioactivity from [^{14}C]Lys-tRNA$_{E.\ coli}$ and from ϵ-Ac-[^{14}C]Lys-tRNA$_{E.\ coli}$ into hot trichloroacetic acid precipitable material in a rabbit reticulocyte cell-free system. Parallel incubations (37°C) contained 0.6 ml of a lysate-master mix-hemin solution and 50 μl of either Lys-tRNA (●) or ϵ-Ac-Lys-tRNA (▲). The concentration and specific activity of the Lys-tRNA were the same as those of the ϵ-Ac-Lys-tRNA, which is characterized in detail in the Experimental Section. Aliquots (25 μl) were removed from the incubations at the times indicated and assayed for hot trichloroacetic acid precipitable radioactivity (Woodward et al., 1974a). Assuming that N^{α}-substituted lysines were not incorporated into protein, 4500 dpm (= 0.94 × 4800) was available for incorporation in each 25-μl aliquot of the ϵ-Ac-Lys-tRNA incubation, while 4800 dpm was available in each 25 μl of the Lys-tRNA incubation. From the plateau levels of incorporation, the efficiency of incorporation of N^{ϵ}-acetyllysine relative to that of lysine is (2300 cpm/4500 dpm) ÷ (3000 cpm/4800 dpm) = 0.82.

Table I: Puromycin Reactivities of Lys-tRNA, ϵ-Ac-Lys-tRNA, and Oligo(Lys)-tRNA.[a]

	[Mg^{2+}] (mM)	Cold Trichloroacetic Acid Precipitable (pmoles)		pmoles Reacted with Puromycin
		−Puromycin	+Puromycin	
Lys-tRNA	10	4.2	3.9	0.3
	30	4.0	3.7	0.3
ϵ-Ac-Lys-tRNA	10	3.1	2.9	0.2
	30	3.9	3.7	0.2
$(Lys)_n$-tRNA	10	10.0	3.6	6.4
$(n \geq 2)$[b]	30	10.1	8.6	1.5

[a] Puromycin reactivity was assayed as described in the Experimental Section. In the absence of ribosomes, no reaction with puromycin occurred. [b] Oligo(Lys)-tRNA was prepared and characterized as described by Menninger and Walker (1974); in this preparation 82% of the lysine was bound to tRNA as oligo(lysine) with a chain length of 2 or greater. Each incubation originally contained 0.54 A_{260} unit of oligo([^{14}C]Lys)-tRNA (118 pmol of Lys/A_{260} unit of tRNA; 76 mCi/mmol of Lys). The above values were calculated assuming an average oligo(lysine) chain length of 5 (Menninger et al., 1970) and a 7% reaction of Lys-tRNA with puromycin (above).

tRNA in the lysate. The results showed that 3–11 A_{260} units of unacylated, unfractionated *E. coli* tRNA per ml of incubation inhibited synthesis less than 25% in a 60-min incubation. Aminoacylation and acetylation of the $tRNA^{Lys}$ to form ϵ-Ac-Lys-tRNA did not markedly alter the effect of the heterologous tRNA.

Parallel lysate incubations, containing either ϵ-Ac-[^{14}C]Lys-tRNA or an equal amount of [^{14}C]Lys-tRNA, were assayed for the incorporation of radioactivity into hot trichloroacetic acid precipitable material. As shown in Figure 3, the extent of incorporation of radioactivity from ϵ-Ac-Lys-tRNA was 82% that from Lys-tRNA. The incorporation was complete within 5 min for both the analogue and the control. At this time the lysate is synthesizing protein at its maximum rate (Woodward et al., 1974a). Based on estimates of the relative abundance of $tRNA^{Lys}$ in *E. coli* (von Ehrenstein, 1967) and in reticulocytes (Smith and McNamara, 1972), the concentration of aminoacylated *E. coli* $tRNA^{Lys}$ in the cell-free incubations was about 70% that of total reticulocyte $tRNA^{Lys}$.

Identification of Radioactive Species Incorporated into Protein. The post-ribosomal supernatant from a large-scale reticulocyte cell-free incubation containing ϵ-Ac-[^{14}C]Lys-tRNA$_{E.\ coli}$ was obtained as described in the Experimental Section. The protein in this supernatant was digested enzymatically to amino acids in order to determine the degree of acetylation of the radioactive lysines incorporated into protein. The resulting digestion mixture was placed on an ion-exchange column and the column was developed under conditions which separate globin peptides (Jones, 1964; Schroeder, 1972; Woodward and Herbert, 1972; Woodward et al., 1974b). Most (79%) of the ^{14}C radioactivity eluted near the void volume of the column, while the [^{3}H]lysine marker eluted much later in the gradient. The ^{14}C radioactivity in the major peak fractions was subsequently analyzed by paper electrophoresis at pH 1.9; this radioactivity coelectrophoresed with the N^{ϵ}-acetyllysine marker. Six percent of the ^{14}C radioactivity eluted with the [^{3}H]lysine marker. The remainder of the ^{14}C was found in small peaks which probably resulted from incompletely digested peptides. Therefore, a minimum of 79% and a maximum of 94% of the lysine incorporated into protein in the reticulocyte cell-free system was acetylated.

Gel Filtration of Post-Ribosomal Supernatant. To determine whether N^{ϵ}-acetyllysine was incorporated into hemoglobin, an aliquot of the post-ribosomal supernatant was analyzed by G-75 Sephadex chromatography. As shown in Figure 4, a large part of the radioactivity in the post-ribosomal supernatant cochromatographed with the hemoglobin absorbance at 410 nm. Thus, the N^{ϵ}-acetyllysine was not only incorporated into complete globin chains, but was present in tetrameric hemoglobin. The small shoulder trailing the major radioactivity peak (fractions 61–70) results from globin dimers (Adamson et al., 1968). The small peak of radioactivity at the void volume may be due either to globin aggregates or to radioactivity associated with residual ribosomes in the post-ribosomal supernatant. The radioactivity in fractions 119–132 is probably due primarily to free N^{ϵ}-acetyllysine and N^{α}-substituted lysines which were hydrolyzed from the tRNA during the incubation and work-up. Nearly all (90%) of the radioactivity placed on the column was recovered in these peaks. The recovered radioactivity was distributed as follows: tetramers, 57%; dimers, 7%; aggregates or ribosome bound, 4%; small molecules, 32%.

Figure 3 shows that the incorporation of N^{ϵ}-acetyllysine into hot trichloroacetic acid precipitable material was 82% that of lysine. Figure 4 shows that 64% of the N^{ϵ}-acetyllys-

ine was incorporated into complete globin chains. It is not surprising that these percentages differ, since it is probable that not all of the Lys-tRNA added to the incubation of Figure 3 transferred its Lys into hot trichloroacetic acid precipitable material. In addition, it is likely that some of the radioactivity which eluted at fractions 119–132 and at the void volume (Figure 4) was in hot trichloroacetic acid precipitable nascent chains.

Discussion

The chemical modification of the ϵ-amino group of Lys-tRNA described in this paper can be carried out with only minor effects on the biological activity of the aminoacyl-tRNA. The sites on $tRNA^{Lys}$ that interact with the cognate synthetase are essentially unimpaired by the exposure of the tRNA to the acetylation reaction conditions. Lys-tRNA and ϵ-Ac-Lys-tRNA have similar message and binding site specificities in their binding to ribosomes, though their ribosomal binding affinities differ, particularly at low Mg^{2+}. The retention of biological activity is further demonstrated by the transfer of N^{ϵ}-acetyllysine from ϵ-Ac-Lys-$tRNA_{E.\ coli}$ into protein in a rabbit reticulocyte lysate system. The highly active cell-free system used in these experiments functions in the initiation of protein synthesis as well as elongation and termination (Woodward et al., 1974a), and provides the most demanding test of the biological integrity of the analogue. In order to transfer its amino acid into protein, the *E. coli* aminoacyl-tRNA analogue must interact successfully with heterologous ribosomes while competing with the full in vivo complement of reticulocyte Lys-tRNA.

As shown in Figure 3, the transfer of N^{ϵ}-acetyllysine into protein was 82% of the transfer of lysine. This is sufficiently high to allow use of this class of analogues as probes of the interactions between aminoacyl-tRNA and other macromolecules involved in protein biosynthesis. The precise cause of the 18% lower incorporation of N^{ϵ}-acetyllysine relative to lysine is not known. One possibility is a lesion in the tRNA of the analogue, such as acetylated tRNA bases or hydrolyzed phosphodiester bonds. Alternatively, a component of the CFS I lysate which recognized the ϵ-Ac-Lys-tRNA as abnormal could have deacylated up to 18% of it prior to incorporation; if so, such an activity might not be observed in a completely homologous system. It is also possible that only a subpopulation of the $tRNA^{Lys}$ was inactivated by the above mechanisms. Finally, the lower incorporation may result from the modification of the lysine moiety. Similarly, it is not known whether the lower ribosomal binding affinity of the analogue relative to Lys-tRNA is due to the acetylation of the ϵ-amino nitrogen of the lysine or to an alteration of the tRNA.

The fact that tRNA exposed to the acetylation procedures exhibits only a slightly diminished ability to be aminoacylated and to participate in protein synthesis suggests that the reaction of *N*-acetoxysuccinimide with functionally important tRNA bases in our procedures is minimal. However, acetylation of the tRNA may occur. Acylation of the tRNA has been observed by investigators using *N*-hydroxysuccinimide esters in reactions of longer duration (10 min to 22 h) than reported here and with other substrate tRNAs. The site(s) of tRNA acylation is uncertain in some of these cases, but cytidine (Friedman, 1972), thiol bases (Schofield et al., 1970), the X base (Friedman, 1973; Nauheimer and Hedgcoth, 1974), and unknown bases (Schofield et al., 1970; Friedman, 1972) have all been suggested as possible

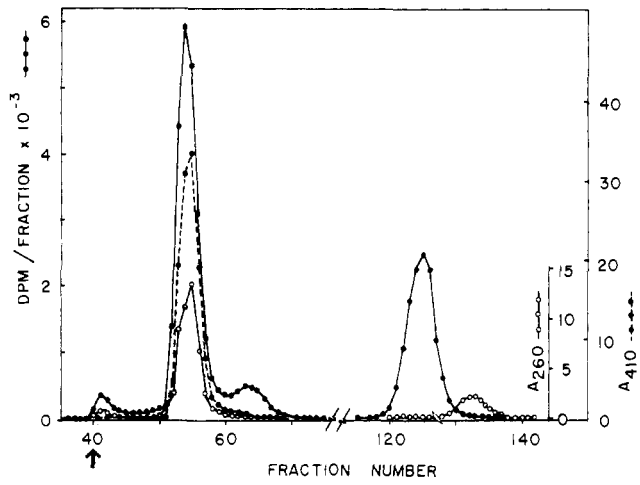

FIGURE 4: Gel filtration of the post-ribosomal supernatant from a reticulocyte cell-free incubation which included ϵ-Ac-[^{14}C-]Lys-$tRNA_{E.\ coli}$. One milliliter of the post-ribosomal supernatant (see the Experimental Section) was layered on a G-75 Sephadex column (Superfine grade; 0.9 cm diam. × 113 cm) equilibrated with a buffer solution containing 10 mM potassium phosphate (pH 6.8), 50 mM KCl, and 0.02% NaN_3. The flow rate was 2.5 ml/hr at room temperature. Fractions of 0.6 ml were collected and assayed for absorbance at 260, 280, and 410 nm (280-nm data not shown). The radioactivity in each fraction was determined with 0.5 ml of the fraction, 0.5 ml of water, and 10.0 ml of Triton scintillator (Menninger et al., 1970); counting efficiencies were determined by internal standardization with [^{14}C]-toluene. The void volume of the column, determined with blue dextran 2000, was 26 ml.

targets. The extent of tRNA acylation varies considerably and appears to depend on the pH of the incubation, the species of tRNA, and the organic solvent used, as well as the time of reaction.

The appearance of N^{ϵ}-acetyllysine in complete globin chains (Figure 4) and the similarity in the incorporation profiles for lysine and N^{ϵ}-acetyllysine (Figure 3) indicate that the ribosome and its associated supernatant factors have no requirements, steric or otherwise, that prevent participation in protein synthesis of an amino acid side chain which is 2–3 Å longer than the side chain of any natural amino acid. The data of Figure 4 also demonstrate that the incorporation of N^{ϵ}-acetyllysine into a protein polymer did not prevent the subsequent completion of the synthesis of that polymer (elongation plus termination). In addition, the gel filtration results show that the presence of some acetylated lysines in the globin polymers was compatible with the association of the globin chains into hemoglobin-like complexes.

The methods reported here make possible the preparation of aminoacyl-tRNA analogues with affinity, photoaffinity, or spectroscopic labels on the lysine side chain. N^{ϵ}-Bromoacetyl-Lys-tRNA and N^{ϵ}-(*N*-methylanthraniloyl)-Lys-tRNA, for example, have been prepared using methods similar to those described here; both analogues show poly(rA)-stimulated binding to ribosomes (A. E. Johnson, unpublished data). The various analogues of Lys-tRNA should prove useful as probes of the ribosomal complex.

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