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Ribosomal Binding and Dipeptide Formation by Misacylated tRNA^{Phe}'s†

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ABSTRACT: Eight structurally modified peptidyl-tRNA^{Phe}'s were employed to study P-site binding and peptide bond formation in a cell-free system involving *Escherichia coli* ribosomes programmed with poly(uridylic acid). It was found that the two analogues (*N*-acetyl-D-phenylalanyl-tRNA^{Phe} and *N*-acetyl-D-tyrosyl-tRNA^{Phe}) containing D-amino acids functioned poorly as donors in the peptidyltransferase reaction and that two *N*-acetyl-L-phenylalanyl-tRNA^{Phe}'s differing from the prototype substrate in that they contained 2′- or 3′-deoxyadenosine at the 3′-terminus failed to form dipeptide at all when L-phenylalanyl-tRNA^{Phe} was the acceptor tRNA. Interestingly, all four of these peptidyl-tRNA's bound to ribosomes to about the same extent as tRNA's that functioned normally as donors in the peptidyltransferase reaction, at least in the absence of competing peptidyl-tRNA species. Two peptidyl-tRNA's lacking an amino group were also tested. In comparison with *N*-acetyl-L-phenylalanyl-tRNA^{Phe} it was found that *trans*-cinnamyl-tRNA^{Phe} and 3-phenylpropionyl-tRNA^{Phe}'s formed dipeptides to the extent of 53 and 80%, respectively, when L-phenylalanyl-tRNA^{Phe} was used as the acceptor tRNA. *N*-Acetyl-β-phenylalanyl-tRNA^{Phe} was found to be the most efficient donor substrate studied. Both isomers transferred *N*-acetyl-β-phenylalanine to L-phenylalanyl-tRNA^{Phe}; the nature of the dipeptides formed in each case was verified by HPLC in comparison with authentic synthetic samples. Further, the rate and extent of peptide bond formation in each case exceeded that observed with the control tRNA, *N*-acetyl-L-phenylalanyl-tRNA^{Phe}.

Peptide bond formation is one of several partial reactions that jointly constitute protein biosynthesis. The peptide bond forming process is catalyzed by peptidyltransferase; in bacteria this activity is an integral part of the 50S ribosomal subunit (Allen & Zamecnik, 1962; Nathans, 1964; Traut & Monro, 1964; Monro, 1967; Harris & Symons, 1973a,b; Symons et al., 1979). Although several studies have attempted to define the structural and spatial parameters requisite for effective participation of aminoacyl- and peptidyl-tRNA's in the peptidyltransferase reaction, most of these have relied on the use of aminoacylated oligonucleotides structurally related to the 3′-terminus of aminoacyl-tRNA (Monro & Marker, 1967; Monro et al., 1968; Mercer & Symons, 1972; Hecht, 1977; Quiggle et al., 1981) or on aminoacyl-tRNA analogues accessible via aminoacyl-tRNA synthetase catalyzed misacylations (Hecht et al., 1974; Chinali et al., 1974; Hecht, 1977; Alford & Hecht, 1978; Pezzuto & Hecht, 1980; Wagner & Sprinzl, 1983).

Recently, we have described a technique ("chemical aminoacylation") in which T4 RNA ligase was employed to couple 2′(3′)-O-acylated pCpA derivatives to tRNA-C_{OH}'s,[1] i.e., tRNA's lacking the 3′-terminal pCpA. The success of the ligation reaction was not a function of the nature of the *O*-acyl group; accordingly, a structurally diverse collection of misacylated tRNA's was prepared (Heckler et al., 1984a,b) and shown to function in ribosome-mediated peptide bond formation (Heckler et al., 1983; Roesser et al., 1986). Described herein are experiments that explore in more detail the structural requirements for ribosomal binding by peptidyl-tRNA analogues, as well as acyl group transfer from the bound species. Key findings include the ability of poly(U)-programmed *Escherichia coli* ribosomes to bind *R* and *S* isomers of *N*-acetylaminoacyl-tRNA's containing α-amino acids and the transfer of simple acyl groups from their 2′(3′)-*O*-acyl-tRNA derivatives to L-phenylalanyl-tRNA^{Phe}. Also studied was *N*-acetyl-β-L-phenylalanyl-tRNA^{Phe}, which was shown to participate in dipeptide formation more quickly and to a greater extent than *N*-acetyl-L-phenylalanyl-tRNA^{Phe}. Remarkably, both enantiomers of β-phenylalanine functioned equally as well in dipeptide formation; the structures of the derived *N*-acetyl-β-phenylalanyl-L-phenylalanines were verified

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[1] Abbreviations: tRNA-C_{OH}, tRNA missing the 3′-terminal cytidine and adenosine moieties; BD-cellulose, benzoylated (diethylaminoethyl)-cellulose; Pipes, 1,4-piperazinediethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-pressure liquid chromatography; DEAE-cellulose, (diethylaminoethyl)cellulose.

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by direct comparison with authentic samples.

Experimental Procedures

Materials. *E. coli* tRNA^{Phe} (specific activity 1200 pmol/A_{260} unit) and poly(U) were purchased from Sigma Chemical Co. T4 RNA ligase was obtained from Pharmacia P-L Biochemicals; 1 unit was defined as the amount of enzyme that catalyzed the formation of 1 nmol of phosphatase-resistant ^{32}P from [5′-^{32}P]oligo(rA) in 30 min at 37 °C. DEAE-cellulose (DE-23) was purchased from Whatman; benzoylated (diethylaminoethyl)cellulose (BD-cellulose) was obtained from Boehringer-Mannheim. [^3H]-L-Phenylalanine (23 Ci/mmol) and [^3H]CTP (20 Ci/mmol) were purchased from New England Nuclear; [^3H]-L-phenylalanine was purified by chromatography on BD-cellulose prior to use. *E. coli* ribosomes and phenylalanyl-tRNA synthetase were isolated as described (Pezzuto & Hecht, 1980). *N*-Acetyl-L-phenylalanyl-tRNA^{Phe} (~3 Ci/mmol) was prepared essentially as described (Rappoport & Lapidot, 1974) and purified by chromatography on BD-cellulose (Heckler et al., 1984a).

Synthesis of N-Acetyl-D,L-β-phenylalanyl-L-phenylalanine. D,L-β-Phenylalanine (400 mg; 2.4 mmol) was dissolved in 2 mL of acetic anhydride. The reaction mixture was heated at 80 °C for 1 h and then concentrated to dryness under diminished pressure. The residue was dissolved in aqueous methanol and lyophilized, affording *N*-acetyl-D,L-β-phenylalanine as a white powder, yield 500 mg (100%): ^1H NMR (CH_3OH-d_4) δ 1.90 (s, 3), 2.78 (d, 2), 5.35 (t, 1), and 7.30 (m, 5).

N-Acetyl-D,L-β-phenylalanine (210 mg; 1.0 mmol), *N*-hydroxysuccinimide (115 mg; 1.0 mmol), and *N*,*N*′-dicyclohexylcarbodiimide (210 mg; 1.0 mmol) were dissolved in 10 mL of ethyl acetate, and the solution was stirred at 25 °C for 24 h. The resulting precipitate of *N*,*N*′-dicyclohexylurea was filtered, and the filtrate was concentrated under diminished pressure. The residue was triturated with acetone, affording *N*-acetyl-D,L-β-phenylalanine *N*-hydroxysuccinimide ester as a white solid, yield 100 mg (33%): ^1H NMR (CH_3OH-d_4) δ 1.95 (s, 3), 2.78 (s, 4), 3.10 (d, 2), 5.44 (t, 1), and 7.34 (m, 5).

N-Acetyl-D,L-β-phenylalanine *N*-hydroxysuccinimide ester (150 mg; 0.5 mmol) and L-phenylalanine (25 mg; 0.15 mmol) were dissolved in 400 μL of *N*,*N*-dimethylformamide. The reaction mixture was stirred at 25 °C for 24 h and then concentrated to dryness under diminished pressure. The residue was applied to a (1.5 × 40 cm) silica gel column, which was washed with $CHCl_3$ and then with $CHCl_3$ containing increasing amounts (5–20%) of CH_3OH. The fractions containing the desired product were concentrated to dryness, affording *N*-acetyl-D,L-β-phenylalanyl-L-phenylalanine as a white solid, yield 35 mg (66%): ^1H NMR (CH_3OH-d_4) δ 1.95 (s, 3), 2.70 (q, 2), 2.97 (q, 1), 3.20 (q, 1), 4.58 (q, 1), 5.32 (t, 1), and 7.15–7.40 (m, 10).

Synthesis of 2′(3′)-O-(N-Acetyl-L-β-phenylalanyl)pCpA. D,L-β-Phenylalanine was analyzed for phenylalanine content by ninhydrin assay; phenylalanine was found to react much more quickly with that reagent than β-phenylalanine at 60 °C. Although standard mixtures of the two amino acids indicated that 0.1% phenylalanine could have been detected reliably, no phenylalanine was apparent in the commercial sample of β-phenylalanine. D,L-β-Phenylalanine was resolved as described by Fischer et al. (1910) by fractional crystallization of the quinidine salt of *N*-formyl-D,L-β-phenylalanine. Following hydrolysis of the *N*-formyl group (10% aqueous HCl, reflux, 1 h), individual enantiomers were obtained as colorless microcrystals from H_2O: $[\alpha]^{20}_D$ +6.71° (*c* 0.87, H_2O) (L-β-phenylalanine); $[\alpha]^{20}_D$ −7.11° (*c* 0.87, H_2O) (D-β-phenylalanine) [cf. Graf and Boeddeker (1958)].

To 50 mg (0.3 mmol) of L-β-phenylalanine was added 0.3 mL of acetic anhydride. The reaction mixture was heated at 80 °C for 1 h and then treated with 1 mL of water. The mixture was concentrated and the residue was dissolved in 0.3 mL of H_2O and cooled, which effected crystallization of *N*-acetyl-L-β-phenylalanine as colorless microcrystals, yield 35 mg (56%), mp 190–192 °C: $[\alpha]^{20}_D$ +85.6° (*c* 1.0, CH_3OH).

N-Acetyl-L-β-phenylalanine (10.4 mg; 0.05 mmol) and 1,1′-carbonyldiimidazole (8.1 mg, 0.05 mmol) were dissolved in 0.2 mL of dimethyl sulfoxide; the solution was stirred at 25 °C for 20 min. To this solution was added 100 A_{260} units (~5 μmol) of pCpA (Heckler et al., 1984a,b), and the reaction mixture was stirred at 25 °C for 6 days. The reaction mixture was applied to a (20 × 20 cm) cellulose TLC plate and developed successively with acetone and then with 5:2:3 1-butanol–acetic acid–H_2O. The derived product formed a UV-active band, R_f 0.55, and was isolated by extraction with 0.01 N HOAc, affording 32 A_{260} units (32%) of diacylated pCpA (Heckler et al., 1984a,b) as a white solid. This material was dissolved in water, adjusted to pH 2 with 1 N HCl, and stirred overnight at 25 °C. Following neutralization to pH 4 with aqueous Na_2CO_3, the solution was concentrated to a small volume and applied to a cellulose TLC plate for purification. Development with 5:2:3 1-butanol–acetic acid–H_2O gave a single major UV-active band, R_f 0.47. The material in this band was isolated by extraction with 0.01 N HOAc, affording 2′(3′)-*O*-(*N*-acetyl-L-β-phenylalanyl)pCpA as a white solid, yield 20 A_{260} units (63%): λ_{max} (0.01 N HOAc) 260 nm; λ_{min} 236 nm.

2′(3′)-*O*-(*N*-Acetyl-D-β-phenylalanyl)pCpA was synthesized analogously, starting from D-β-phenylalanine.

Synthesis of Cytidylyl(3′→5′)-2′-deoxyadenosine 5′-Monophosphate (pCp2′dA). A solution of collidine (0.4 mL), tetrahydrofuran (1.4 mL), and methyl phosphorodichloridite (Martin & Pizzolato, 1950; Ogilvie et al., 1980) (74 μL; 0.78 mmol) was cooled to −78 °C and treated dropwise with a solution of 440 mg (0.6 mmol) of N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(monomethoxytrityl)cytidine (Ogilvie et al., 1979) in 2.6 mL of tetrahydrofuran over a period of 35 min. The combined solution was stirred at −78 °C for an additional 30 min and then treated dropwise at −78 °C with a solution containing 192 mg (0.54 mmol) of N^6,$O^{3'}$-dibenzoyl-2′-deoxyadenosine (Ogilvie, 1973) in 2.6 mL of tetrahydrofuran. The combined solution was stirred at −78 °C for 30 min and then at 25 °C for 15 min. The reaction mixture was then treated with I_2 (152 mg; 0.6 mmol) in 1.8 mL of 1:1:1 pyridine–water–tetrahydrofuran, and stirring was continued for 15 min at 25 °C. The reaction mixture was concentrated to dryness, and the residue was dissolved in $CHCl_3$, washed with aqueous $NaHSO_3$ and water, and then dried (Na_2SO_4). Concentration under diminished pressure afforded a syrup that was purified by flash chromatography on silica gel (Still et al., 1978). Elution with 10:9:1 ethyl acetate–$CHCl_3$–CH_3OH afforded the fully protected dinucleotide as a colorless foam, yield 240 mg (40%): silica gel TLC R_f 0.35 (10:9:1 ethyl acetate–$CHCl_3$–CH_3OH).

Detritylation was effected by dissolving 1.03 g (0.81 mmol) of the dinucleoside monophosphate in 125 mL of a 7:3 $CHCl_3$–C_2H_5OH mixture containing 1% benzenesulfonic acid. The solution was maintained at 4 °C for 15 h and then treated with 1.8 mL of pyridine and concentrated to dryness. The residue was dissolved in $CHCl_3$, and the solution was washed

with water, dried (Na_2SO_4), and concentrated, yielding a foam. This material was purified by preparative TLC (silica gel, development with 10:9:1 ethyl acetate–$CHCl_3$–CH_3OH), affording methyl N^4-benzoyl-2′-*O*-(*tert*-butyldimethylsilyl)-cytidylyl(3′→5′)-N^6,$O^{3'}$-dibenzoyl-2′-*O*-deoxyadenosine as a chromatographically homogeneous, colorless foam, yield 345 mg (43%): λ_{max}^{EtOH} 264 and 230 nm; (partial) ^1H NMR [$CDCl_3$, $(CH_3)_4Si$] δ 0.13 (s, 3), 0.16 (s, 3), 0.87 (s, 9), 3.8–3.85 (d, 3), 7.45–8.2 (m, 16), 8.33 (s, 1), 8.45 (s, 1), and 8.77 (d, 1); silica gel TLC R_f 0.17 (10:9:1 ethyl acetate–$CHCl_3$–CH_3OH).

The detritylated dinucleoside monophosphate (87 mg; 87 μmol) was dissolved in 2 mL of cold tetrahydrofuran and added dropwise over a period of 30 min to a cold solution consisting of 0.5 mL of collidine, 1 mL of tetrahydrofuran, and 50 μL (0.54 mmol) of $POCl_3$. The reaction mixture was stirred at −78 °C for 1 h and then at 0 °C for 30 min. The reaction mixture was then treated with 0.5 mL of water, stirred at 0 °C for an additional 30 min, and concentrated to dryness under diminished pressure. The residue was dissolved in $CHCl_3$, washed with H_2O, dried (Na_2SO_4), and concentrated. The residue was treated with 2.5 mL of 1:2:2 thiophenol–triethylamine–dioxane, and the solution was stirred at 25 °C for 1 h. The solution was concentrated to dryness, and the residual oil was dissolved in ethyl acetate and extracted exhaustively with water. The aqueous layer was concentrated under diminished pressure in 20 mL of concentrated NH_4OH and stirred at 25 °C for 17 h. The solution was concentrated and the residue was dissolved in 10 mL of water, washed with ether, and concentrated. The residue was purified by preparative TLC (silica gel, development with 7:1:2 2-propanol–NH_4OH–H_2O). Elution of material from the main band (7:30 2-propanol–H_2O) afforded 2′-*O*-(*tert*-butyldimethylsilyl)cytidylyl(3′→5′)-2′-deoxyadenosine 5′-monophosphate as a white solid, yield 550 A_{260} units (30%): λ_{max} 265 nm; silica gel TLC R_f 0.56 (7:1:2 2-propanol–NH_4OH–H_2O). The ^1H NMR spectrum (D_2O) indicated the disappearance of signals due to the benzoyl and methyl phosphate groups [formerly at δ 7.45–8.2 and 3.8–3.85, respectively (vide supra)].

The silylated dinucleotide (550 A_{260} units) was dissolved in 6 mL of tetrahydrofuran containing 1 M tetrabutylammonium fluoride. The reaction mixture was stirred at 25 °C for 24 h and then diluted with 100 mL of water and applied to a DEAE-cellulose column (1.5 × 13 cm; HCO_3^- form). The column was washed with 100 mL of water and then with 50 mL of 0.5 M $NH_4^+HCO_3^-$ to effect elution of the desired product. The appropriate fractions were desalted by repeated evaporation of portions of water and then dissolved in water and lyophilized. Cytidylyl(3′→5′)-2′-deoxyadenosine 5′-monophosphate was obtained as a white powder, yield 515 A_{260} units (94%): λ_{max} 262 nm; silica gel TLC R_f 0.29 (development with 7:1:2 2-propanol–NH_4OH–H_2O): cellulose TLC R_f 0.14 (development with 5:2:3 2-propanol–HOAc–H_2O). The ^1H NMR spectrum reflected the disappearance of upfield resonances corresponding to the *tert*-butyldimethylsilyl group.

Synthesis of 3′-O-(N-Acetyl-L-phenylalanyl)pCp2′dA. A solution containing 22 mg (106 μmol) of *N*-acetyl-L-phenylalanine and 18 mg (110 μmol) of 1,1′-carbonyldiimidazole in 0.5 mL of dry dimethylsulfoxide was stirred at 25 °C for 20 min and then treated with 480 A_{260} units (24 μmol) of pyridinium pCp2′dA. Stirring was continued at 25 °C for 40 h, and the reaction mixture was then added dropwise to 6.5 mL of dry acetone and centrifuged to isolate the precipitate. The product was dissolved in a small amount of water and applied to a preparative cellulose TLC plate. Development with 2:2:1 acetone–EtOH–H_2O gave two bands of material, which were isolated by washing with 100 mL of H_2O. Concentration gave two compounds: the less polar was the N^4,$O^{3'}$-diacylated pCp2′dA derivative [58 A_{260} units; cellulose TLC R_f 0.73 (development with 2-propanol–NH_4OH–H_2O); λ_{max} 306 and 252 nm]; the more polar was the N^4-acyl pCp2′dA derivative [90 A_{260} units; cellulose TLC R_f 0.67 (development with 2-propanol–NH_4OH–H_2O); λ_{max} 306 and 252 nm].

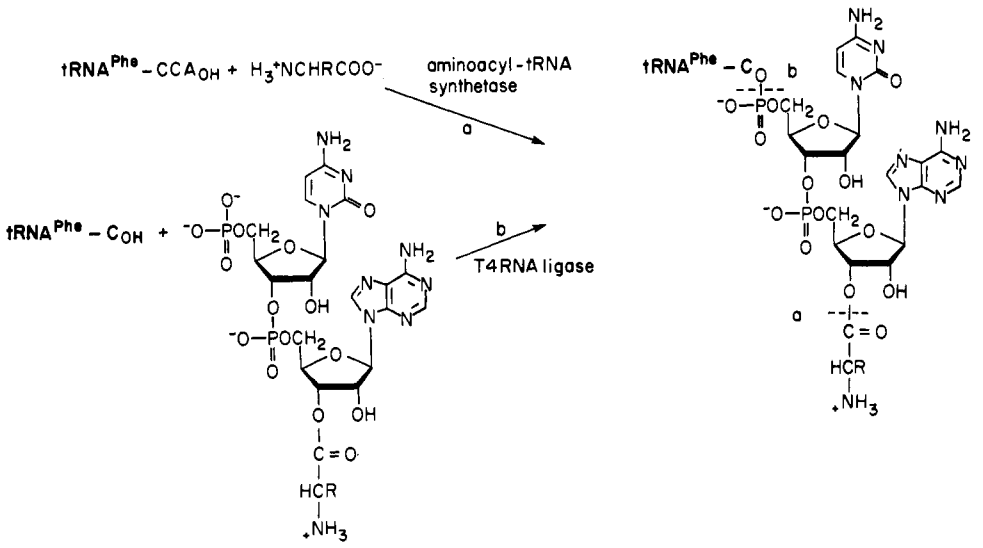
N^4,$O^{3'}$-Bis(*N*-acetyl-L-phenylalanyl)pCp2′dA (45 A_{260} units) was dissolved in 1 mL of 0.01 N HCl. The solution was maintained at 25 °C for 24 h and then neutralized on a Dowex 50W column (0.5 × 2.5 cm; Na^+ form). The column was washed with 10 mL of water, and the eluate was concentrated to a small volume and applied to a preparative cellulose TLC plate. Development with 1-butanol saturated with 10% HOAc, and then with 5:2:3 1-butanol–HOAc–H_2O, afforded 3′-*O*-(*N*-acetyl-L-phenylalanyl)pCp2′dA as a white solid, yield 4.6 A_{260} units: λ_{max} 262 nm.

Aminoacyl-tRNA^{Phe}-[^{3}H]CCA Analogues. *E. coli* tRNA^{Phe}-[^{3}H]C_{OH} was prepared from intact tRNA^{Phe} essentially as described (Heckler et al., 1983, 1984a), except that [^{3}H]CTP was employed for reconstitution of the 3′-terminus of the venom-treated tRNA. The N-acetylaminoacyl pCpA derivatives were obtained by chemical synthesis as described (Heckler et al., 1984a,b) and then ligated to the 3′-terminus of *E. coli* tRNA^{Phe}-[^{3}H]C_{OH} via the agency of T4 RNA ligase. Purification by successive chromatographic purifications on DEAE-cellulose and BD-cellulose (Heckler et al., 1983, 1984a) afforded the desired *N*-acetylaminoacyl-tRNA^{Phe}-[^{3}H]CCA's in 20–40% overall yields.

Peptidyltransferase-Mediated Dipeptide Formation. The misacylated *N*-acetylaminoacyl-tRNA^{Phe}'s were utilized in poly(U)-directed peptidyltransferase assays essentially as described (Heckler et al., 1983); authentic *N*-acetyl-L-phenylalanyl-tRNA^{Phe} was employed as a reference sample in each case. Ten picomoles of *N*-acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe} or a misacylated *N*-acetylaminoacyl-tRNA^{Phe} was incubated at 25 °C in 90 μL (total volume) of 100 mM Tris-HCl, pH 7.8, containing 150 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 50 μg of poly(U), and 3.7 A_{260} units of *E. coli* ribosomes. After 15 min, the incubation mixture was treated with 30 pmol of L-phenylalanyl-tRNA^{Phe} or [^{3}H]-L-phenylalanyl-tRNA^{Phe} (3.5 Ci/mmol) that had been prepared by simultaneous 15-min incubation (25 °C) of 25 μL of 90 mM NH_4–Pipes, pH 7.0, containing 100 mM KCl, 0.5 mM EDTA, 2.5 mM ATP, 15 mM $MgCl_2$, 5 μM phenylalanine, 0.05 A_{260} unit of tRNA^{Phe}, and 2 μL of phenylalanyl-tRNA synthetase. The combined solution was maintained at 25 °C for an additional 15 min and then treated with 100 μL of 2 M NaOH. Analysis for dipeptide formation was carried out by neutralization of the reaction mixture with 2 M HOAc, dilution of the sample to 0.5 mL with 50 mM NaOAc, pH 4.5, containing 0.45 M NaCl, and application of the diluted sample to a 1-mL BD-cellulose column that had been equilibrated at 25 °C with the same buffer. The column was washed with several milliliters of 50 mM NaOAc, pH 4.5, containing 0.45 M NaCl and 5% ethanol (to effect removal of excess [^{3}H]phenylalanine or *N*-acetyl[^{3}H]phenylalanine) (1.0-mL fractions), and then with 3 mL of 50 mM NaOAc, pH 4.5, containing 0.45 M NaCl, 30% ethanol, and 32% formamide to effect elution of the formed dipeptides. The fractions were used for determination of radioactivity by addition to 10 mL of a xylene-based scintillation fluid.

Effect of Mn^{2+} on Ribosomal Binding of N-Acetylaminoacyl-tRNA^{Phe}'s and Participation in Dipeptide Formation.

Scheme I: Illustration of Bonds Established during Preparation of Aminoacyl-tRNA's by Enzymatic (a) and "Chemical" (b) Activation



Reaction mixtures containing 90 μL (total volume) of 100 mM Tris-HCl, pH 7.8, 2.5 A_{260} units of *E. coli* 70S ribosomes, 3 mM $MgCl_2$, 150 mM NH_4Cl, 0.05 mg/mL poly(U), 1 mM 2-mercaptoethanol, and 0–10 mM $MnCl_2$ were maintained at 4 °C for 3 min. Ten picomoles of *N*-acetyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} was added to the reaction mixture, which was maintained at 4 °C for 15 min and then filtered through nitrocellulose filters. The filters were washed with 5 mL of 100 mM Tris-HCl, pH 7.8, containing 150 mM NH_4Cl, 1 mM 2-mercaptoethanol, 3 mM $MgCl_2$, and the same concentration of $MnCl_2$ used initially; the dried filters were used for determination of radioactivity.

Dipeptide formation was carried out in 90 μL (total volume) of 100 mM Tris-HCl, pH 7.8, containing 150 mM NH_4Cl, 1 mM 2-mercaptoethanol, 0.05 mg/mL poly(U), 3 mM $MgCl_2$, 0–10 mM $MnCl_2$, and 1.5 A_{260} units of *E. coli* ribosomes. Ten picomoles of *N*-acetyl[^{3}H]-L-phenylalanine was added to the reaction mixture, and the ribosomal complex was permitted to form for 15 min at 4 °C. Twenty picomoles of phenylalanyl-tRNA^{Phe} was added, and dipeptide formation was allowed to proceed at 25 °C for 15 min. The reaction mixture was quenched by treatment with 90 μL of 1 M NaOH; after 30 min at 25 °C, the solution was neutralized with acetic acid and applied to a BD-cellulose column for analysis of dipeptide content (vide supra).

The ability of *N*-acetyl-D-tyrosyl-tRNA^{Phe} and *N*-acetyl-D-phenylalanyl-tRNA^{Phe} to form dipeptides in a poly(U)-programmed system containing [^{3}H]-L-phenylalanyl-tRNA^{Phe} was assayed as indicated above by using 1 mM Mn^{2+} and 3 mM Mg^{2+}.

Results

Of the nine peptidyl-tRNA^{Phe} analogues studied (Chart I), *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (**1**) and *N*-acetyl-L-phenylalanyl-tRNA^{Phe}-CC3′dA (**9**) were prepared by chemical acetylation of tRNA's that had been activated enzymatically with phenylalanyl-tRNA synthetase. The remaining seven tRNA's studied were obtained via T4 RNA ligase mediated coupling of the appropriate acylated pCpA derivative with tRNA^{Phe}-C$_{OH}$, i.e., by "chemical aminoacylation" (Heckler et al., 1983, 1984a,b) (Scheme I). The yields of the purified tRNA's prepared by this method ranged from 20 to 65%.

The ability of individual peptidyl-tRNA^{Phe} analogues to participate in peptide bond formation as donor substrates was assayed in a cell-free system. High salt washed *E. coli* ribosomes (Pezzuto & Hecht, 1980) were programmed with poly(uridylic acid), and 10 pmol of either *N*-acetyl[^{3}H]-L-phenylalanyl-tRNA^{Phe} or peptidyl-tRNA^{Phe} analogue was preincubated and allowed to prebind to the ribosomes for 15 min at 25 °C. Thirty-fifty picomoles of either L-phenylalanyl-tRNA^{Phe} or [^{3}H]-L-phenylalanyl-tRNA^{Phe} was then added to the reaction mixture, and dipeptide formation was allowed to proceed for 15 min. Aminoacyl-tRNA bonds were then hydrolyzed by the addition of NaOH, and dipeptide formation was quantified following purification of the products on BD-cellulose.

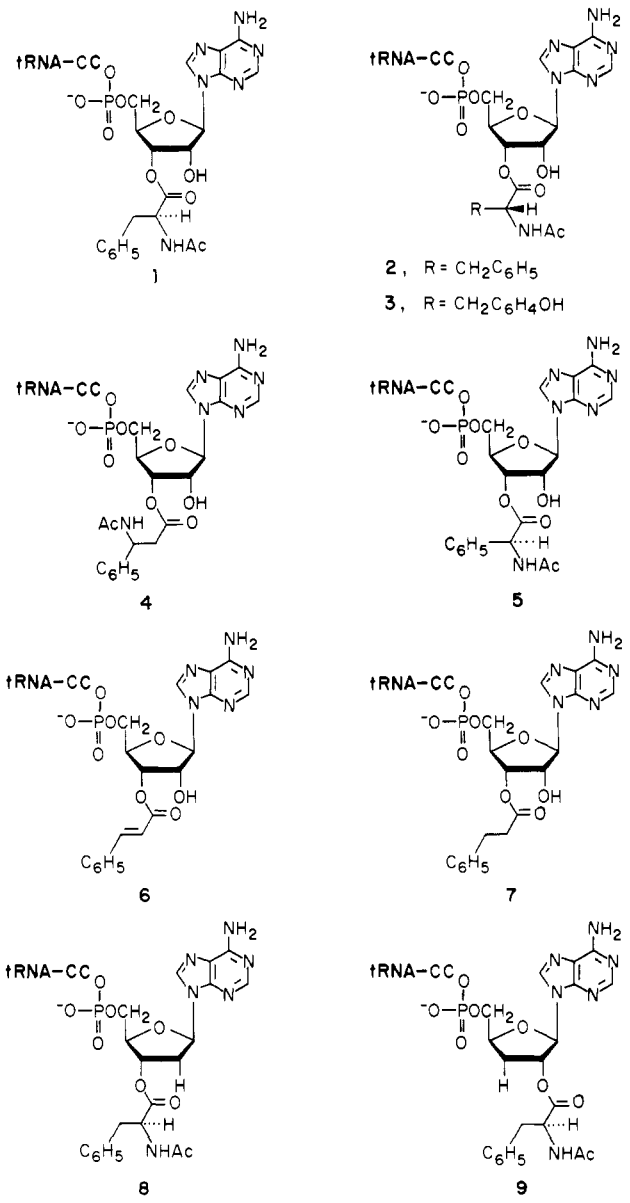
Table I: Peptidyltransferase-Mediated Dipeptide Synthesis Using Analogues of *N*-Acetyl-L-phenylalanyl-tRNA^{Phe} [a]

peptidyl-tRNA^{Phe} analogue	isolated yield of dipeptide: pmol/A_{260} unit of ribosomes	isolated yield of dipeptide: % relative to peptidyl-tRNA **1**
N-acetyl-L-phenylalanyl-tRNA^{Phe} (**1**)[b]	1.5	100
N-acetyl-D-phenylalanyl-tRNA^{Phe} (**2**)[c]	0.1	7
N-acetyl-D-tyrosyl-tRNA^{Phe} (**3**)[c]	0.2	13
N-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} (**4**)[c]	1.7	110
N-acetyl-D,L-phenylglycyl-tRNA^{Phe} (**5**)[c]	1.4	93
trans-cinnamyl-tRNA^{Phe} (**6**)[c]	0.8	53
3-phenylpropionyl-tRNA^{Phe} (**7**)[c]	1.2	80
N-acetyl-L-phenylalanyl-tRNA^{Phe}-CC2′dA (**8**)[c]	0	0
N-acetyl-L-phenylalanyl-tRNA^{Phe}-CC3′dA (**9**)[b]	0	0

[a] Carried out as described under Experimental Procedures. [b] Prepared by activation of tRNA^{Phe} with phenylalanine in the presence of *E. coli* phenylalanyl-tRNA synthetase, followed by chemical acetylation with *N*-acetoxysuccinimide. [c] Prepared by "chemical aminoacylation" (Heckler et al., 1983, 1984a).

As illustrated in Table I, the peptidyl-tRNA^{Phe} analogues *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} (**4**), *N*-acetyl-D,L-phenylglycyl-tRNA^{Phe} (**5**), 3-phenylpropionyl-tRNA^{Phe} (**7**), and to a slightly lesser extent *trans*-cinnamyl-tRNA^{Phe} (**6**), were utilized efficiently as donor substrates in the peptidyl-transferase reaction. The yields of dipeptides obtained with these peptidyl-tRNA^{Phe} analogues were 53–110% of that obtained in a control reaction that employed *N*-acetyl-L-phenylalanyl-tRNA^{Phe} as the donor substrate. Interestingly, however, *N*-acetyl-D-phenylalanyl-tRNA^{Phe} (**2**) and *N*-acetyl-D-tyrosyl-tRNA^{Phe} (**3**) gave only small amounts of dipeptide products, and the peptidyl-tRNA analogues ter-

Chart I: Structures of Peptidyl-tRNA^{Phe} Analogues Studied[a]



[a] The 2′(3′)-*O*-acyl moieties are shown arbitrarily on the 3′-position of ribose.

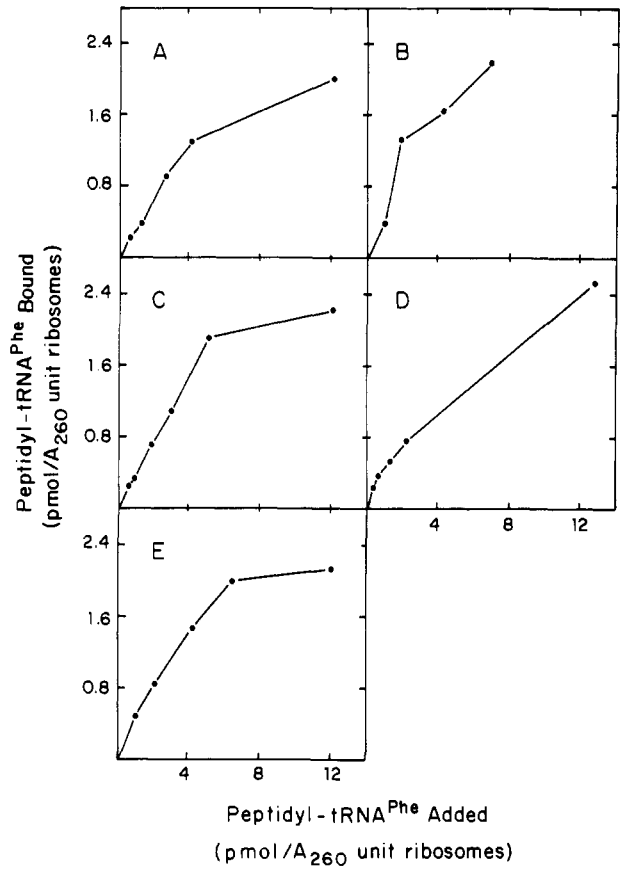


FIGURE 1: Binding of peptidyl-tRNA^{Phe} analogues to *E. coli* ribosomes programmed with poly(uridylic acid). Ribosomal binding was measured for authentic *N*-acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe} (panel A), chemically aminoacylated *N*-acetyl-D-phenylalanyl-tRNA^{Phe}-[^{3}H]CCA (panel B), *N*-acetyl-D-tyrosyl-tRNA^{Phe}-[^{3}H]CCA (panel C), *trans*-cinnamyl-tRNA^{Phe}-[^{3}H]CCA (panel D), and *N*-acetyl-L-phenylalanyl-tRNA^{Phe}-[^{3}H]CC2′dA (panel E). Assays were run in 50-μL reaction mixtures (total volume) containing 100 mM Tris-HCl, pH 7.8, 150 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 30 μg of poly(U), 2.5 A_{260} units of *E. coli* ribosomes, and varying amounts of the peptidyl-tRNA^{Phe} analogues. The reaction mixtures were maintained at 25 °C for 15 min and then diluted with 0.5 mL of cold 100 mM Tris-HCl, pH 7.8, containing 150 mM NH_4Cl, 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol and immediately filtered on nitrocellulose filters (Millipore HAWP). The filters were rinsed three times with the same buffer, air-dried, and used for determination of radioactivity.

minating with 2′- or 3′-deoxyadenosine (**8** and **9**) produced no detectable *N*-acetylphenylalanylphenylalanine under the assay conditions.

To determine whether the inability of certain peptidyl-tRNA's to function as donors in the peptidyltransferase reaction was due to some functional constraint or simply to lack of ribosomal binding per se, the ability of several of the peptidyl-tRNA's to bind to *E. coli* ribosomes was measured directly. This was done in a nitrocellulose filter binding assay by the use of peptidyl-tRNA's having [^{3}H]cytidine in the acceptor stem. As shown in Figure 1, measurement of the amount of peptidyl-tRNA^{Phe} analogues bound to *E. coli* ribosomes as a function of the amount of peptidyl-tRNA employed in the assay indicated that the ribosomal binding of peptidyl-tRNA analogues that functioned poorly in peptide bond formation (**2**, **3**, and **8**; panels B, C, and E, respectively, of Figure 1) did not differ dramatically from those that functioned well as donors (**1** and **6**; panels A and D, respectively). Half-maximal saturation of available ribosomal sites occurred for each of the analogues at ~3–5 pmol of tRNA analogue/A_{260} unit of 70S ribosomes. Further, when the maximal binding for each peptidyl-tRNA analogue was measured by using 30 pmol of tRNA for each A_{260} unit of ribosomes, all of the peptidyl-tRNA's studied bound to the ribosomes to a similar extent (Table II). To determine whether the binding of individual peptidyl-tRNA^{Phe} analogues might differ *qualitatively*, we also measured the ability of three different analogues to bind to *E. coli* ribosomes in the presence of *N*-acetyl[^{3}H]-L-phenylalanyl-tRNA^{Phe}. As shown in Table II, there were significant differences in the amounts of individual peptidyl-tRNA^{Phe} analogues required to inhibit the binding of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} by 50%. Thus 3-phenylpropionyl-tRNA^{Phe} (**7**), which was an efficient donor in the peptidyltransferase reaction (Table I), was quite effective in diminishing the binding of peptidyl-tRNA **1** to *E. coli* ribosomes. On the other hand, *N*-acetyl-D-phenylalanyl-tRNA^{Phe} (**2**) and *N*-acetyl-L-phenylalanyl-tRNA^{Phe}-CC2′dA (**8**) were less effective in inhibiting the ribosomal binding of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (**1**), in parallel with their lesser activities as substrates in the peptidyltransferase reaction.

The factor-independent peptidyltransferase assays described above were dependent on high (10 mM) concentrations of

Table II: Binding of *N*-Acetyl-L-phenylalanyl-tRNA^{Phe} Analogues to Poly(U)-Programmed *E. coli* Ribosomes[a]

peptidyl-tRNA^{Phe} analogue	ribosomal binding (pmol/A_{260} unit of ribosomes)	competitive binding[b] ([analogue]:[1])
N-acetyl-L-phenylalanyl-tRNA^{Phe} (**1**)	3.0	
N-acetyl-D-phenylalanyl-tRNA^{Phe} (**2**)	2.3	2.5:1
N-acetyl-D-tyrosyl-tRNA^{Phe} (**3**)	3.0	
trans-cinnamyl-tRNA^{Phe} (**6**)	2.5	
3-phenylpropionyl-tRNA^{Phe} (**7**)		1.5:1
N-acetyl-L-phenylalanyl-tRNA^{Phe}-CCA2'dA (**8**)	2.8	6:1

[a] The binding assays were carried out essentially as described in the legend to Figure 1 by using 0.20 A_{260} unit of *E. coli* ribosomes, 12 μg of poly(U), and 6 pmol of peptidyl-tRNA^{Phe} analogue. [b] Approximate concentration of peptidyl-tRNA^{Phe} analogue required to reduce the extent of ribosomal binding of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} by 50%. The assays were run essentially as described in the legend to Figure 1 by using 2.0 A_{260} units of ribosomes, 7 pmol of *N*-acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe}, and 0–50 pmol of peptidyl-tRNA^{Phe} analogue; the incubation mixtures were maintained at 25 °C for 20 min prior to filtration.

Table III: Metal Ion Dependence of *N*-Acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe} Binding to *E. coli* Ribosomes[a]

metal ion	concn (mM)	ribosomal binding (pmol of tRNA/A_{260} unit of ribosomes)	metal ion	concn (mM)	ribosomal binding (pmol of tRNA/A_{260} unit of ribosomes)
Mg^{2+}	3	0.8	Mn^{2+}[b]	0	0.8
	10	2.2		1	2.0
				5	2.0
				10	2.0

[a] Carried out as described under Experimental Procedures by using 2.5 pmol of *E. coli* ribosomes and 10 pmol of *N*-acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe}. [b] Carried out in the presence of 3 mM $MgCl_2$ in addition to the indicated amounts of $MnCl_2$.

Mg^{2+}. It has been shown that Mn^{2+} can replace Mg^{2+} in such peptidyltransferase reactions (Pestka, 1972). The use of Mn^{2+} in place of Mg^{2+} has also been employed to decrease the fidelity of RNA- and DNA-dependent DNA polymerases (Sirover & Loeb, 1977; Loeb & Mildvan, 1981; Fersht et al., 1983; El-Deiry et al., 1984). Experiments were carried out to determine whether or not the in vitro system employed here could be made Mn^{2+} dependent and, if so, what the effect of Mn^{2+} would be on the utilization of peptidyl-tRNA^{Phe} analogues that were not utilized as donors in peptide bond formation in the presence of Mg^{2+}. As shown in Table III and Figure 2, at 3 mM Mg^{2+} concentration the binding of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} to *E. coli* ribosomes was incomplete and little dipeptide formation was observed. However, in the presence of 3 mM Mg^{2+} plus 1 mM Mn^{2+} the ribosomal binding of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} was essentially the same as that obtained with 10 mM Mg^{2+}, and *N*-acetyl-L-phenylalanyl-L-phenylalanine was obtained to the extent of 1.6 pmol/A_{260} unit of ribosomes. Accordingly, the peptidyl-tRNA^{Phe} analogues *N*-acetyl-D-tyrosyl-tRNA^{Phe} and *N*-acetyl-D-phenylalanyl-tRNA^{Phe}, which did not participate efficiently in peptidyltransferase reactions at 10 mM Mg^{2+}, were utilized as peptidyl-tRNA^{Phe}'s in peptidyltransferase reactions containing 3 mM Mg^{2+} and either 1, 3, or 10 mM Mn^{2+}. As shown for *N*-acetyl-D-tyrosyl-tRNA^{Phe} in the presence of 1 mM Mn^{2+} [Figure 1 of the supplementary material (see paragraph at end of paper regarding supplementary material)], Mn^{2+} was unable to activate these normally inefficient donor substrates to participate in peptide bond formation under any of the conditions tested.

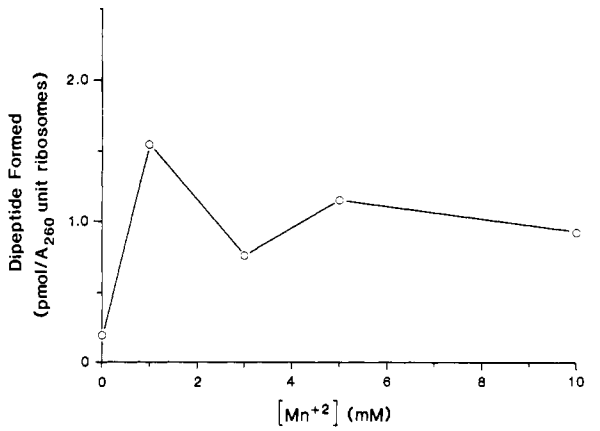


FIGURE 2: Dipeptide formation as a function of $[Mn^{2+}]$. Peptidyltransferase reactions were run with *E. coli* *N*-acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe} and L-phenylalanyl-tRNA^{Phe} in a poly(U)-dependent system that utilized *E. coli* ribosomes. Dipeptide formation was carried out at various Mn^{2+} concentrations in the presence of 3 mM Mg^{2+}. The assay is described under Experimental Procedures.

It has been shown that several peptidyl-tRNA^{Phe} analogues prepared by chemical aminocylation participated efficiently as donor substrates in in vitro peptidyltransferase reactions (Table I). The measurement of donor efficiency of these peptidyl-tRNA^{Phe} analogues involved assaying the amount of dipeptide formed by acyl group transfer to L-phenylalanyl-tRNA^{Phe} within 15 min, which was assumed to be sufficient to allow complete reaction. In order to develop another parameter of dipeptide formation to permit the analysis of peptidyl-tRNA analogue function, we examined the time course of dipeptide formation using L-phenylalanyl-tRNA^{Phe} as the acceptor tRNA and the two peptidyl-tRNA^{Phe} analogues (**1** and **4**) found to be the most efficient donors.

Fifteen picomoles of either *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (**1**) or *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} (**4**) was prebound to 70S ribosomes, and [^{3}H]-L-phenylalanyl-tRNA^{Phe} was then added to the reactions to initiate dipeptide formation. The reactions were quenched at predetermined times after admixture of [^{3}H]-L-phenylalanyl-tRNA^{Phe} by the addition of NaOH. Sodium hydroxide quenches the peptidyltransferase reaction by hydrolyzing the aminoacyl and peptidyl-tRNA^{Phe} bonds; Na^+ has also been shown to be a potent inhibitor of peptidyltransferase (Pestka, 1972). Formation of the two dipeptides as a function of time is shown in Figure 3. As anticipated in the original assays, 15 min proved to be sufficient to permit essentially complete dipeptide formation, at least for these efficient donors. Also confirmed (cf. Table I) was the greater extent of dipeptide formation obtained with *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} as compared with the "normal" donor **1**. As shown in Figure 3, there was a lag time of at least 2 min before significant amounts of *N*-acetyl-L-phenylalanyl-L-phenylalanine were formed. For *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe}, the lag time for dipeptide formation was slightly, but reproducibly, less than that for *N*-acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe}. Peptide bond formation with the β-amino acid was 70% complete after 2-min reaction time. The difference in the kinetics of peptide bond formation could not have been due to differences in the binding of the two peptidyl-tRNA's to the ribosome, as nitrocellulose filter binding assays indicated that binding of *N*-acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe} was complete after 15 min. These results indicate that *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} (**4**)

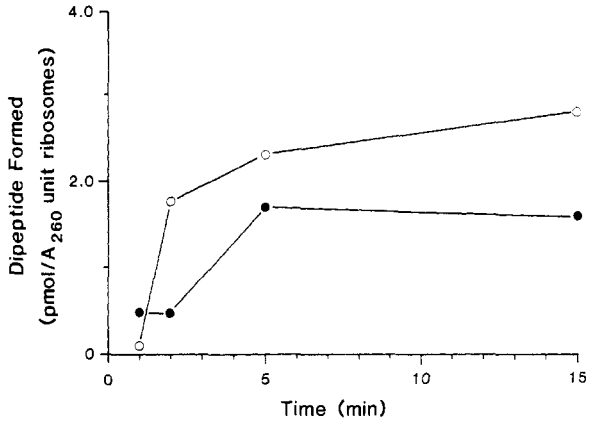


FIGURE 3: Time course of dipeptide formation. *E. coli* *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (●) and *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} (○) were bound to *E. coli* ribosomes that had been programmed with poly(U); the extent of dipeptide formation was measured as a function of time following addition of L-phenylalanyl-tRNA^{Phe} as an acceptor. Assays were run in 90 μL of 100 mM Tris-HCl, pH 7.8, containing 150 mM NH_4Cl, 1 mM 2-mercaptoethanol, 0.05 mg/mL of poly(U), and 10 mM $MgCl_2$. Fifteen picomoles of *N*-acetyl-L-phenylalanyl-tRNA^{Phe} or *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} was added to 1.8 A_{260} units of *E. coli* ribosomes, and the complexes were permitted to form at 4 °C for 15 min. Thirty picomoles of *E. coli* [^{3}H]-L-phenylalanyl-tRNA^{Phe} was then added to initiate dipeptide formation. The reaction mixtures were maintained at 25 °C, and aliquots were removed after 1, 2, 5, and 15 min and quenched by the addition of 90 μL of 1 M NaOH. After the hydrolysis had been permitted to proceed at 25 °C for 30 min, the individual aliquots were neutralized with acetic acid and analyzed for dipeptide formation (see Experimental Procedures).

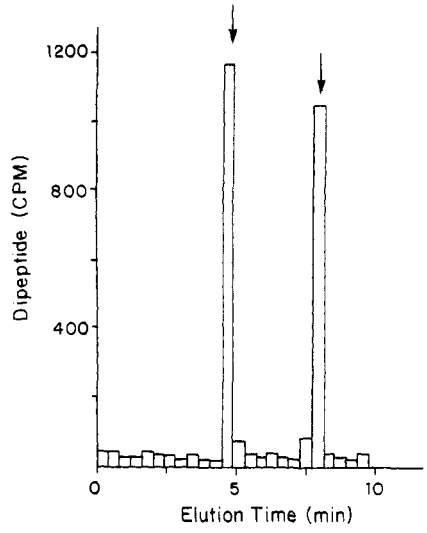


FIGURE 4: Analysis of dipeptides formed with *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} as a donor, and [^{3}H]-L-phenylalanyl-tRNA^{Phe} as an acceptor, in a peptidyltransferase reaction. Analysis of the isolated dipeptide products by C_{18} reverse-phase HPLC indicated the formation of two radiolabeled products that comigrated with the authentic diastereomers (arrows) of *N*-acetyl-D,L-β-phenylalanyl-L-phenylalanine. The dipeptides were formed by using poly(U)-programmed *E. coli* ribosomes, separated from unreacted amino acids on BD-cellulose as described under Experimental Procedures, and desalted prior to HPLC analysis. The HPLC analysis was carried out on a Rainin Microsorb C_{18} column; elution was with 19% CH_3CN in 25 mM Et_3N–HOAc, pH 7.0.

was utilized more efficiently as a donor in the peptidyltransferase reaction than was *N*-acetyl-L-phenylalanyl-tRNA^{Phe}.

The high efficiency of utilization of bound *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} (**4**) in peptide bond formation (Table I and Figure 3) suggested that both D and L isomers of this peptidyl-tRNA might well participate in peptide bond formation, in spite of the lack of efficient utilization of peptidyl-tRNA's **2** and **3**. This was tested directly by HPLC analysis of putative *N*-acetyl-D,L-β-phenylalanyl-[^{3}H]-L-phenylalanine, formed in a peptidyltransferase reaction involving *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} (**4**) and [^{3}H]-L-phenylalanyl-tRNA^{Phe}. As shown in Figure 4, an authentic diastereomeric mixture of this dipeptide was readily separated by C_{18} reverse-phase HPLC; two products having mobilities identical with those of authentic diastereomers resulted from the peptidyltransferase reaction.

To confirm the nature of the products formed in Figure 4, we resolved D,L-β-phenylalanine into its optical antipodes and prepared *N*-acetyl-L-β-phenylalanyl-tRNA^{Phe} (27% yield) and *N*-acetyl-D-β-phenylalanyl-tRNA^{Phe} (24% yield). Each of these peptidyl-tRNA^{Phe} analogues was employed as a donor in the peptidyltransferase reaction, using [^{3}H]-L-phenylalanyl-tRNA^{Phe} as the acceptor species. The products of both reactions were analyzed by BD-cellulose chromatography. Both the D and L isomers were found to be efficient donors, producing dipeptide to the extent of ~130%, relative to a control that employed *N*-acetyl-L-phenylalanyl-tRNA^{Phe}. The results obtained with *N*-acetyl-D-β-phenylalanyl-tRNA^{Phe} are shown in Figure 2 of the supplementary material.

DISCUSSION

The present study employs peptidyl-tRNA^{Phe} analogues to dissect certain facets of ribosome binding and peptide bond formation. The source of the activated tRNA analogues is worthy of comment. As illustrated in Scheme I, there are two methods presently available for tRNA activation. One of these involves aminoacyl-tRNA synthetase catalyzed attachment of an amino acid to an intact tRNA in a process that constitutes the normal cellular source of aminoacyl-tRNA's. Because the overall fidelity of protein biosynthesis cannot exceed that of tRNA activation (Crick, 1958; Hoagland, 1959), the accurate pairing of a tRNA with its cognate amino acid would seem to be essential. Nonetheless, aminoacyl-tRNA synthetase catalyzed activation can potentially provide misacylated tRNA's when abnormal reaction conditions are employed in a cell-free system (Ebel et al., 1973; Giegé et al., 1974), or by a process thought to obtain in vivo when certain cognate amino acids are absent (Ebel et al., 1973; Giegé et al., 1974). Clearly, misacylated tRNA's can also result from modification of the aminoacyl moieties of tRNA's activated with their cognate amino acids (Chapeville et al., 1962; Fahnestock & Rich, 1971a,b). It may also be mentioned that, with the identification of the specific nucleotides that determine tRNA isoacceptor specificity (McClain & Nicholas, 1987), it has also been possible to alter tRNA identity (Normanly et al., 1986).

The second method of tRNA activation involves the bacteriophage T4 RNA ligase mediated coupling of 2′(3′)-*O*-(aminoacyl) derivatives of pCpA with tRNA's from which the last two nucleotides have been removed. As shown in Scheme I, while the bond formed by this process is different than that resulting from tRNA activation by an aminoacyl-tRNA synthetase, where the aminoacyl moiety on the pCpA derivative is cognate to the tRNA being modified, the ultimate product is the same. When this method for tRNA activation is employed, the potential for misacylation is clear, since T4 RNA ligase does not recognize the *O*-acyl substituent (Heckler et al., 1984a,b).

By the use of peptidyl-tRNA^{Phe} analogues, we demonstrated earlier that *N*-acetyl-L-tyrosyl-tRNA^{Phe} functioned nearly as well as the phenylalanine derivative in peptide bond formation but that neither D isomer formed significant amounts of di-

peptide when L-phenylalanyl-tRNA^{Phe} was employed as the acceptor tRNA (Heckler et al., 1983). Presently, we extend these observations in a few important ways. First, it was observed that *trans*-cinnamyl-tRNA^{Phe} (**6**) and 3-phenylpropionyl-tRNA^{Phe} (**7**) both functioned reasonably well as donors in the peptidyltransferase reaction, demonstrating that the presence of the *N*-acetylamino group was not required for acyl group transfer. On the other hand, the consistently greater efficiency of *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} as a peptidyl-tRNA substrate (110–130%, relative to *N*-acetyl-L-phenylalanyl-tRNA^{Phe}) argues both that the *N*-acetylamino group can have a positive effect on peptidyl-tRNA function (cf. **4** vs **7**, Table I) and that the normal position of this group on C$^{\alpha}$ may well be nonoptimal for facility of peptide bond formation. Direct comparison of the amount of *N*-acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe} bound to the P site of *E. coli* ribosomes with the amount of *N*-acetyl-L-phenylalanyl-L-phenylalanine formed upon subsequent admixture of phenylalanyl-tRNA^{Phe} suggests that 70–90% of the *N*-acetyl-L-phenylalanyl-tRNA^{Phe} initially bound to the ribosomal P site is eventually used in the peptidyltransferase reaction. Accordingly, the greater efficiency of dipeptide formation observed with *N*-acetyl-D,L-β-phenylalanyl-tRNA^{Phe} may simply reflect utilization of a greater proportion of the peptidyl-tRNA initially bound. However, we cannot exclude the possibility that translocation of the ribosomes may occur under our experimental conditions (Bergemann & Nierhaus, 1983), permitting participation of the ribosome in additional peptidyltransferase reactions.

Also of interest were the results obtained when the ribosomal binding of certain peptidyl-tRNA^{Phe} analogues was measured. As shown in Table II and Figure 1, all of the tRNA's studied bound to *E. coli* 70S ribosomes to about the same extent; this was true both for maximal extent of binding and for binding as a function of added peptidyl-tRNA in the presence of a constant amount of ribosomes. In contrast, for three tRNA's studied (**2**, **7**, and **8**, Table II) the ability to inhibit the ribosomal binding of *N*-acetyl-[^{3}H]-L-phenylalanyl-tRNA^{Phe} varied significantly and was in the same order as the substrate activities of the three species in peptide bond formation. Although the ribosomal binding of (the individual isomers of) *N*-acetyl-D,L-phenylglycyl-tRNA^{Phe} (**5**) was not measured directly, the ability of this tRNA mixture to function almost as well as *N*-acetyl-L-phenylalanyl-tRNA^{Phe} (**1**) in peptide bond formation argues that the L isomer of **5** must be bound to the ribosomes selectively, as the D isomer is presumably a poor donor in peptide bond formation (cf. tRNA's **2** and **3**, Table I). In the aggregate, these data suggest that the ribosomal binding of tRNA's that function well in peptide bond formation may sometimes differ qualitatively or quantitatively from those that do not.

In addition to their ability to bind to the ribosomal P site in an appropriate fashion, it must be anticipated that those peptidyl-tRNA^{Phe} analogues that participate in peptide bond formation also fulfill some minimum requirements with regard to chemical structure and reactivity. As indicated in Table I, replacement of the 3′-terminal adenosine moiety in *N*-acetyl-L-phenylalanyl-tRNA^{Phe} with either 2′-deoxyadenosine (tRNA **8**) or 3′-deoxyadenosine (tRNA **9**) resulted in species that would not participate in peptide bond formation to any detectable extent. This observation was consistent with earlier reports (Hecht et al., 1974; Chinali et al., 1974); presumably this is due, at least in part, to the lower chemical reactivity anticipated for such analogues (Zachau, 1960; Zachau & Karau, 1960; Sprinzl & Cramer, 1973, Chinault et al., 1977).

The minimal activity of *N*-acetyl-D-phenylalanyl-tRNA^{Phe} (**2**) and *N*-acetyl-D-tyrosyl-tRNA^{Phe} (**3**) as donors in the peptidyltransferase reaction parallels findings for the donor activities of aminoacylated tRNA fragments (Quiggle et al., 1981), as well as the differences noted for D- and L-tyrosyl-tRNA^{Tyr}'s in ribosomal A-site studies (Yamani et al., 1981). In contrast, both the D and L isomers of *N*-acetyl-β-phenylalanyl-tRNA^{Phe} were more efficient as donors than was *N*-acetyl-L-phenylalanyl-tRNA^{Phe} itself. This suggests strongly that, at least for tRNA's in the ribosomal P site, stereochemistry at C$^{\beta}$ is not an important determinant of substrate activity. Since peptidyltransferase has recently been demonstrated to be capable of catalyzing the formation of peptide analogues having altered connectivity (Roesser et al., 1986), it may not be unreasonable to anticipate that aminoacyl-tRNA analogues having the amino group at positions other than C$^{\alpha}$ could function as acceptor tRNA's in protein biosynthesis.

The efficiency of utilization of modified peptidyl-tRNA's as donors in the peptidyltransferase reaction has two implications for studies employing misacylated tRNA's for the elaboration of modified proteins. First, because peptidyltransferase appears largely insensitive to substituents at positions other than C$^{\alpha}$, it should be possible to utilize misacylated tRNA's to incorporate molecular probes at the N-terminus of derived proteins. Second, given the remarkable behavior of *N*-acetyl-D- and *N*-acetyl-L-β-phenylalanyl-tRNA^{Phe}'s as donors in peptide bond formation, it seems logical to anticipate that were an analogous β-aminoacyl-tRNA to function successfully in the ribosomal A site as an acceptor tRNA, the resulting peptidyl-tRNA (i.e., having altered connectivity between the two amino acids proximal to the tRNA) would continue to function in protein biosynthesis following translocation to the ribosomal P site.

It must be emphasized that the results obtained here employed an in vitro system for peptide bond formation that utilized a homopolymer as a template and an analogue of the normal initiator tRNA. Further, while the ability of at least one misacylated tRNA to compete with the cognate species has been established in an analogous system (Pezzuto & Hecht, 1980) and the greater donor efficiency of *N*-acetyl-β-phenylalanyl-tRNA^{Phe}, as compared with *N*-acetyl-L-phenylalanyl-tRNA^{Phe}, seems unequivocal, it must be noted that the present studies differ from those encountered in vivo in a few important ways. The most obvious include the lack of competing peptidyl-tRNA's in most of the experiments run and the absence of a natural mRNA and relevant protein factors required for protein biosynthesis in vivo.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Figure 1, showing attempted enhancement of dipeptide formation in the presence of Mn^{2+}, and Figure 2, showing dipeptide formation using *N*-acetyl-D-β-phenylalanyl-tRNA^{Phe} as the peptidyl-tRNA (3 pages). Ordering information is given on any current masthead page.

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