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Participation in Protein Biosynthesis of Transfer Ribonucleic Acids Bearing Altered 3′-Terminal Ribosyl Residues†

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ABSTRACT: Properties of tRNA^{Phe}-C-C-3′dA and tRNA^{Phe}-C-C-A$_{oxi-red}$ from yeast, both aminoacylated exclusively on the 2′-hydroxyl group of the terminal adenosine, were studied in partial reactions of protein biosynthesis using the *Escherichia coli* system. Unlike Phe-tRNA^{Phe}-C-C-A$_{oxi-red}$, Phe-tRNA^{Phe}-C-C-3′dA is enzymatically bound to ribosomes by EF-T and causes EF-T-dependent GTP hydrolysis. EF-T-dependent binding and GTP hydrolysis thus require an intact ribose ring, but no vicinal hydroxyl group on the terminal adenosine; the position of the amino acid is either unessential or only the 2′ isomer is used. Phe-tRNA^{Phe}-C-C-3′dA and, less efficiently, Phe-tRNA^{Phe}-C-C-A$_{oxi-red}$ react as acceptors with Ac-Phe-tRNA^{Phe}-C-C-A forming dipeptides, but the rates of peptide-bond formation are much slower than that observed with Phe-tRNA^{Phe}-C-C-A. The 2′ isomer of aminoacyl-tRNA can therefore accept the peptidyl residue; however, the presence of a vicinal hydroxyl group and intact ribose ring greatly influence the rate of the reaction. The Phe-tRNA^{Phe} analogs inhibit peptide chain elongation, Phe-tRNA^{Phe}-C-C-3′dA being a more effective inhibitor because it competes with EF-T-directed binding of Phe-tRNA^{Phe}-C-C-A to the ribosomal A site. Phe-tRNA^{Phe}-C-C-3′dA bound enzymatically to the A site is displaced from ribosomes by Phe-tRNA^{Phe}-C-C-A, indicating that the -C-C-3′dA-Phe terminus of this tRNA^{Phe} analog does not bind as tightly as the unmodified Phe-tRNA^{Phe} to the acceptor region of the peptidyltransferase center. The *N*-acetyl derivatives of Phe-tRNA^{Phe}-C-C-3′dA and Phe-tRNA^{Phe}-C-C-A$_{oxi-red}$ do not function as donor either in the puromycin reaction or in dipeptide formation with Phe-tRNA^{Phe}-C-C-A bound enzymatically to the A site. The presence of vicinal hydroxyl group or the transacylation to the 3′ position is thus required for this reaction. Both modified Ac-Phe-tRNAs bind to the ribosomal P site and thus block the initiation of poly(phenylalanine) synthesis in the presence of Phe-tRNA^{Phe}-C-C-A. Since it has been shown that the 3′ isomer of an aminoacyl-tRNA analog can also function as acceptor of the peptidyl residue, it is now evident that both the 2′- and the 3′-aminoacyl-tRNAs are substrates in the A site of the peptidyltransferase center. From these findings one can draw the conclusions on the required conformation of the aminoacyl residue during peptide-bond formation. A model is proposed in which the α-amino group of the amino acid occupies the same spatial position in both 2′- and 3′-aminoacylated tRNA.

The hydroxyl groups of the 3′-terminal ribose of tRNA are directly involved in various steps of protein biosynthesis, *e.g.*, aminoacylation, T-factor recognition, and peptide-bond formation (see review of Lucas-Lenard and Lipmann, 1971). Aminoacyl and peptidyl residues are bound by an ester linkage to the 2′- or 3′-hydroxyl group of the ribose residue of the terminal adenosine and exchange rapidly between those two *cis*-diol positions (Wolfenden *et al.*, 1964; McLaughlin and Ingram, 1965; Griffin *et al.*, 1966). This transacylation might also occur during the various steps of protein biosynthesis. Yeast tRNA^{Phe} missing either the 2′- or the 3′-hydroxyl group of the terminal ribose has been prepared (Sprinzl *et al.*, 1973); only the tRNA^{Phe}-C-C-3′dA[1] is aminoacylated by phenylalanyl-tRNA synthetase whereas the tRNA^{Phe}-C-C-2′dA is a competitive inhibitor for this enzyme (Sprinzl and Cramer, 1973). This shows that the 2′-hydroxyl group is the reactive site for the enzymatic aminoacylation of tRNA. In Phe-tRNA^{Phe}-C-C-3′dA the amino acid is fixed on the 2′-hydroxyl group and cannot undergo transacylation as in native tRNA^{Phe}-C-C-A

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[1] Abbreviations used are: EF-G, elongation factor G; EF-T, mixture of elongation factors Tu and Ts; tRNA^{Phe}-C-C-A, native phenylalanine transfer RNA; Phe-tRNA^{Phe}, phenylalanyl-tRNA^{Phe}; Ac-Phe-tRNA^{Phe}, *N*-acetylphenylalanyl-tRNA^{Phe}; tRNA^{Phe}-C-C-3′dA, tRNA^{Phe} lacking the 3′-hydroxyl group of the terminal ribose, prepared by enzymatic incorporation of 3′-deoxyadenosine (cordycepin) into tRNA^{Phe}-C-C; tRNA^{Phe}-C-C-A$_{oxi-red}$, tRNA^{Phe} lacking the C2′–C3′ bond of the terminal ribose, prepared by periodate oxidation and subsequent borohydride reduction of tRNA^{Phe}-C-C-A; F, formycin, 7-amino-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine.

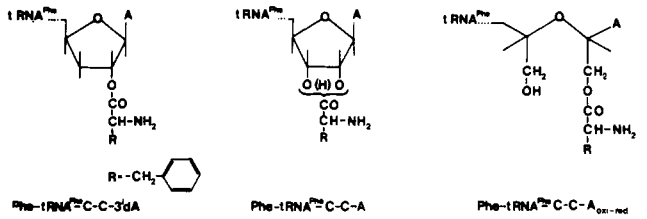

FIGURE 1: Chemical structure of the 3′ terminus of Phe-tRNA^{Phe} species. In Phe-tRNA^{Phe}-C-C-A the aminoacyl residue can be bound to the 2′- or 3′-hydroxyl group.

(Figure 1). Phe-tRNA^{Phe}-C-C-3′dA fails to function in poly(U)-directed poly(phenylalanine) synthesis (Sprinzl and Cramer, 1973). Similarly tRNA^{Phe}-C-C-A$_{oxi-red}$ in which the C2′–C3′ bond of the 3′-terminal ribose is cleaved can be aminoacylated (Cramer *et al.*, 1968; von der Haar *et al.*, 1971) and is inactive in protein biosynthesis (Ofengand and Chen, 1972). Phe-tRNA^{Phe}-C-C-A$_{oxi-red}$ (Figure 1) is not recognized by elongation factor Tu (Chen and Ofengand, 1970). These properties could be explained by the absence of the *cis*-diol group, thus blocking transacylation, and, in the case of tRNA^{Phe}-C-C-A$_{oxi-red}$, they could also be related to a change of conformation in the -C-C-A end caused by opening the ribose ring (Maelicke *et al.*, 1974).

In this communication we compare Phe-tRNA^{Phe}-C-C-3′dA and Phe-tRNA^{Phe}-C-C-A$_{oxi-red}$ in various reactions of protein biosynthesis in order to investigate the role of the intact ribose ring, the requirements for the amino acid position (2′ or 3′) and a possible transacylation from 2′- to 3′-hydroxyl group.

Experimental Section

Materials. tRNA^{Phe}-C-C-A from yeast was prepared from commercial bulk tRNA (Boehringer, Mannheim) according to Schneider *et al.* (1972). It accepted 1470 pmol of phenylalanine/A_{260} unit of tRNA. tRNA^{Phe}-C-C-3′dA was prepared as described previously (Sprinzl *et al.*, 1973) and was free of tRNA^{Phe}-C-C-A as could be shown by 3′-end group analysis (Sprinzl *et al.*, 1970). Its phenylalanine acceptance was 1485 pmol/A_{260} unit of tRNA. tRNA^{Phe}-C-C-A$_{oxi-red}$ was obtained as described (Cramer *et al.*, 1968; von der Haar *et al.*, 1971; Chen and Ofengand, 1970; Ofengand and Chen, 1972) and accepted 1420 pmol of phenylalanine/A_{260} unit of tRNA. Prior to preparative aminoacylation, tRNA^{Phe}-C-C-A$_{oxi-red}$ and tRNA^{Phe}-C-C-3′dA were treated with sodium periodate under conditions which quantitatively oxidized native tRNA^{Phe}-C-C-A (Cramer *et al.*, 1968; von der Haar *et al.*, 1971; Chen and Ofengand, 1970; Ofengand and Chen, 1972); traces of native tRNA^{Phe}-C-C-A would be thereby eliminated. tRNA (5 A_{260} units) was incubated 30 min at 37° in a solution containing 150 mM Tris-HCl buffer (pH 7.65), 200 mM KCl, 50 mM $MgSO_4$, 1 mM ATP, 0.02 mM labeled or unlabeled phenylalanine, 35 mM serum albumin, 7 mM mercaptoethanol, and 500 units/ml of homogeneous phenylalanyl-tRNA synthetase from yeast (E.C. 6.1.1.20) with a specific activity of 1820 units/mg (von der Haar, 1973). The pH was then adjusted to 4.5 with sodium acetate buffer and the reaction mixture was treated with the same volume of chloroform–isoamyl alcohol (5:2). The aqueous phase was passed through a Sephadex G-25 column developed with 1 mM sodium acetate buffer (pH 4.5). Fractions of the excluded peak were concentrated by evaporation at reduced pressure and the aminoacylated tRNAs were precipitated with ethanol. The phenylalanyl-tRNA synthetase from yeast was free of nuclease and tRNA nucleotidyltransferase activities as shown by its inability to aminoacylate tRNA^{Phe}-C-C and tRNA^{Phe}-C-C-2′dA, respectively (Sprinzl *et al.*, 1973). Formation of Phe-tRNA^{Phe}-C-C-A during the enzymatic aminocylation of modified tRNA^{Phe} species could therefore be excluded. tRNA^{Phe} from *E. coli*, accepting 1150 pmol of phenylalanine/A_{260} unit of tRNA, was purified from a commercial tRNA preparation (Schwarz) and aminoacylated with unlabeled phenylalanine (Chinali and Parmeggiani, 1973a). Phe-tRNAs were N-acetylated by the method of Haenni and Chapeville (1966).

Three-times NH_4Cl-washed ribosomes, homogeneous EF-T (EF-Tu + EF-Ts) and homogeneous EF-G were prepared from *E. coli* BT2^r as described (Parmeggiani, 1968); 1 A_{260} unit of ribosomes was assumed to equal 25 pmol; 1 μg of EF-G and EF-T was assumed to correspond to 12 and 15 pmol, respectively (Sander *et al.*, 1972).

Poly(U), GTP, and ATP were purchased from Boehringer (Mannheim, BRD), [^{14}C]phenylalanine (50 or 513 Ci per mol) and [^{3}H]phenylalanine (20,000Ci/mol) from the Radiochemical Centre (Amersham, U. K.). [γ-^{32}P]GTP prepared by the method of Glynn and Chappell (1964) was purified by chromatography on DEAE-cellulose (Whatman DE52) (Sander *et al.*, 1972).

Methods. Binding of [^{14}C]Phe-tRNA^{Phe} species to ribosomes was measured by filtration on nitrocellulose filters (Millipore HAWP 0.45 μm). GTP hydrolysis was determined as liberation of P_i (Parmeggiani, 1968). Poly(U)-directed poly(phenylalanine) synthesis was measured as incorporation of [^{14}C]phenylalanine into hot trichloroacetic acid insoluble material using Whatman No. 3MM paper filters (Chinali and Parmeggiani, 1973a). Large excesses of ATP, [^{14}C]phenylalanine, and phenylalanyl-tRNA synthetase from yeast were present in the *in vitro* system to hold constant the concentration of Phe-tRNA^{Phe}-C-C-A, when the inhibition of poly(phenylalanine) synthesis by tRNA^{Phe} analogs were studied. Under the experimental conditions used, all added tRNA^{Phe} species were completely aminoacylated in less than 2 min at 30°.

Poly(U)–ribosome complexes were prepared by incubating ribosomes and a saturating amount of poly(U) (4 μg/A_{260} unit of ribosomes) in standard buffer with 10 mM $MgCl_2$ for 5 min at 30°. Standard buffer contained 60 mM Tris-HCl (pH 7.8), 30 mM KCl, 30 mM NH_4Cl, and 2 mM dithiothreitol.

In some experiments, EF-T-directed binding of Phe-tRNA^{Phe} species to ribosomes was measured after incubation (10 min, 30°) of poly(U)–ribosomes with tRNA^{Phe}-C-C-A in order to prevent the nonenzymatic binding of Phe-tRNA^{Phe} species to the ribosomal P site (Watanabe, 1972).

Ac-Phe-tRNA^{Phe}-C-C-A–poly(U)–ribosome complex used to test acceptor activities of native and modified Phe-tRNA^{Phe} was prepared by incubation (10 min, 30°) of poly(U)–ribosomes with Ac-Phe-tRNA^{Phe}-C-C-A from *E. coli* (40 pmol/A_{260} unit of ribosomes) in standard buffer with 10 mM $MgCl_2$ (Chinali and Parmeggiani, 1973b). Except when indicated, the complex was isolated prior to use by centrifugation (3 hr, 45,000 rpm, Spinco 50 Ti rotor) through 5 ml of 8% sucrose in standard buffer with 5 mM $MgCl_2$.

Acceptor activity was determined by measuring formation of Ac-Phe-[^{14}C]Phe after enzymatic or nonenzymatic binding of [^{14}C]Phe-tRNA^{Phe} species to Ac-Phe-tRNA^{Phe}–poly(U)–ribosome. After binding reaction the ribosomal complexes were freed from unbound tRNA either by gel filtration on Sepharose 4B (Pharmacia) column (8 × 0.25 cm) or by filtration on Millipore filter. In the first case, ribosomal complexes were precipitated with ice-cold 0.2 M acetic acid and treated with 0.05 ml of 0.3 M NaOH for 1 hr at 30°. In the second case, filters were incubated for 4 hr at room temperature in 1 ml of 0.2 M NH_4OH and washed with 0.5 ml of 0.2 M NH_4OH, and the

TABLE I: Activity of Phe-tRNA^{Phe} Species in EF-T-Directed Binding to Ribosomes and EF-T-Dependent GTP Hydrolysis.[a]

Phe-tRNA^{Phe} Species Added	[^{14}C]Phe-tRNA^{Phe} Species Bound to Ribosomes (pmol)	[γ-^{32}P]GTP Hydrolyzed (pmol)
Expt I (10 min; 0°)		
[^{14}C]Phe-tRNA^{Phe}–C–C–A	16.0 (2.3)[b]	22.7 (2.1)[c]
[^{14}C]Phe-tRNA^{Phe}–C–C–3′dA	15.4 (3.7)[b]	18.2 (2.0)[c]
[^{14}C]Phe-tRNA^{Phe}–C–C–A$_{oxi\text{-}red}$	3.3 (3.3)[b]	0 (0.1)[c]
Expt II (5 min; 30°)		
[^{14}C]Phe-tRNA^{Phe}–C–C–A	19.0 (1.7)[b]	25.3 (1.1)[c]
[^{14}C]Phe-tRNA^{Phe}–C–C–3′dA	16.8 (1.8)[b]	20.8 (2.8)[c]
[^{14}C]Phe-tRNA^{Phe}–C–C–A$_{oxi\text{-}red}$	1.2 (1.1)[b]	0.6 (1.7)[c]

[a] 32.5 A_{260} units of ribosomes, 120 μg of poly(U), and 1800 pmol of non-aminoacylated tRNA^{Phe} from *E. coli* were incubated 10 min at 30° in 0.9 ml of standard buffer with 10 mM $MgCl_2$. Aliquots of 50 μl were added to 50 μl of standard buffer with 10 mM $MgCl_2$ containing 2 μg of EF-T, 140 pmol of [γ-^{32}P]GTP (480 Ci/mol), and 50 pmol of [^{14}C]Phe-tRNA^{Phe} species (513 Ci/mol). Samples were incubated for 10 min at 0° (expt I) or for 5 min at 30° (expt II) and then analyzed for [^{14}C]Phe-tRNA^{Phe} species bound to ribosomes and for [γ-^{32}P]GTP hydrolyzed. Nonenzymatic binding of [^{14}C]Phe-tRNA^{Phe} species was determined in parallel by omitting EF-T. Controls for EF-T-GTPase activity were performed by substituting [^{14}C]Phe-tRNA^{Phe} species with 70 pmol of the corresponding non-aminoacylated tRNA. Values were corrected for aminoacylated tRNA retained on filters in the absence of ribosomes (0.8–1.2 pmol) and for GTP hydrolyzed when tRNA^{Phe} species from yeast were omitted (3.2 pmol in expt I and 5.8 pmol in expt II, respectively). [b] In the absence of EF-T. [c] In the presence of non-aminoacylated tRNA^{Phe} species.

combined solutions, containing 96–99% of the radioactive material originally present on filters, were concentrated under reduced pressure. Aliquots of the hydrolysates obtained by these procedures were analyzed by descending paper chromatography using the solvent system 1-butanol saturated with 2 M NH_4OH (Lucas-Lenard and Lipmann, 1967). After development, chromatograms were dried, cut in 1.5- or 2-cm strips and analyzed for radioactivity.

Donor activity of Ac-[^{14}C]tRNA^{Phe} species were assayed either by the puromycin reaction (Chinali and Parmeggiani, 1973a) or by the formation of Ac-[^{14}C]Phe-Phe in the presence of unlabeled Phe-tRNA^{Phe}-C-C-A. In the latter case Ac-[^{14}C]Phe-tRNA^{Phe} species were incubated for 10 min at 30° with poly(U)–ribosomes and then Phe-tRNA^{Phe}-C-C-A from *E. coli* was bound enzymatically to the A site. The resulting ribosomal complexes were isolated by gel filtration on Sepharose 4B, hydrolyzed with 0.3 M NaOH, and analyzed by descending paper chromatography for dipeptide formation as already described.

Radioactive samples were measured in a toluene scintillation fluid containing 5 g of 2,5-diphenyloxazole/l. Efficiencies of the Packard 3380 scintillation spectrometer for ^{14}C were 65% with paper filters and 78% with Millipore filters. In double-labeling experiments using Millipore filters, efficiencies were 10% for ^{3}H and 8% for ^{14}C in the first channel and 0% for ^{3}H and 51% for ^{14}C in the second channel. For further experimental details, see legends of the figures.

Results

Phe-tRNA^{Phe}-C-C-3′dA and Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ are inactive in the overall process of *in vitro* poly(U)-directed poly(phenylalanine) synthesis (Sprinzl and Cramer, 1973; Ofengand and Chen, 1972). In order to investigate which step of the elongation process was inhibited by these structural modifications of tRNA, we have examined some of the partial reactions involved.

EF-T-Directed Binding of Phe-tRNA^{Phe} Species to Ribosomes. During protein biosynthesis binding of aminoacyl-tRNAs to ribosomes is catalyzed by EF-T in the presence of GTP; concomitantly with binding, GTP is hydrolyzed to GDP and P_i (*cf.* Lucas-Lenard and Lipmann, 1971).

Table I compares the activities of native and modified tRNAs in EF-T-dependent binding and GTPase reactions. Phe-tRNA^{Phe}-C-C-3′dA showed activities close to that of Phe-tRNA^{Phe}-C-C-A whereas the binding of Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ to ribosomes was not stimulated by EF-T, as already observed (Chen and Ofengand, 1970; Ofengand and Chen, 1972), nor did this modified tRNA stimulate EF-T-dependent GTPase activity. These results point out the essential role of the intact 3′-terminal ribose ring for the interaction between aminoacyl-tRNA and EF-Tu.

The effect of Mg^{2+} concentration on the enzymatic or nonenzymatic binding of these Phe-tRNA^{Phe} species is shown in Figure 2. EF-T-directed binding of [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA to ribosomes was similar to that of [^{14}C]Phe-tRNA^{Phe}-C-C-A at 10–14 mM Mg^{2+} but lower at 4–8 mM Mg^{2+}. This indicates a requirement of a higher Mg^{2+} concentration for the interaction of the modified Phe-tRNA^{Phe} with EF-TU and/or with the ribosomal A site (see below). Binding of Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ was not stimulated by EF-T at the Mg^{2+} concentrations tested as also indicated by the results of Table I.

Acceptor Activity of Modified Phe-tRNA^{Phe} Species. Acceptor activity was assayed using ribosomes carrying Ac-Phe-tRNA^{Phe} from *E. coli* bound nonenzymatically in the presence of 10 mM Mg^{22+}. Ac-Phe-tRNA^{Phe} from *E. coli* was preferred to Ac-Phe-tRNA^{Phe} from yeast because optimal conditions for its nonenzymatic binding to ribosomal P site are better known (Lucas-Lenard and Lipmann, 1967; Chinali and Parmeggiani, 1973b). In the experiments illustrated in Figure 3, [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA was bound enzymatically to the ribosomal A site in the presence of 10 mM Mg^{2+}, while [^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ was bound nonenzymatically to the same site in the presence of 15 mM Mg^{2+}. Formation of Ac-Phe-[^{14}C]Phe was observed with both Phe-tRNA^{Phe} analogs. The possibility that formation of dipeptides was caused by contamination with phenylalanyl-tRNA synthetase catalyzing the exchange of [^{14}C]phenylalanine from modified tRNA^{Phe} to *E. coli* tRNA^{Phe} was excluded since addition of a large excess of unlabeled phenylalanine and of ATP affected neither the amount of [^{14}C]Phe-tRNA^{Phe} species bound to ribosomes nor the acceptor activity. tRNA^{Phe}-C-C-3′dA used in these experi-

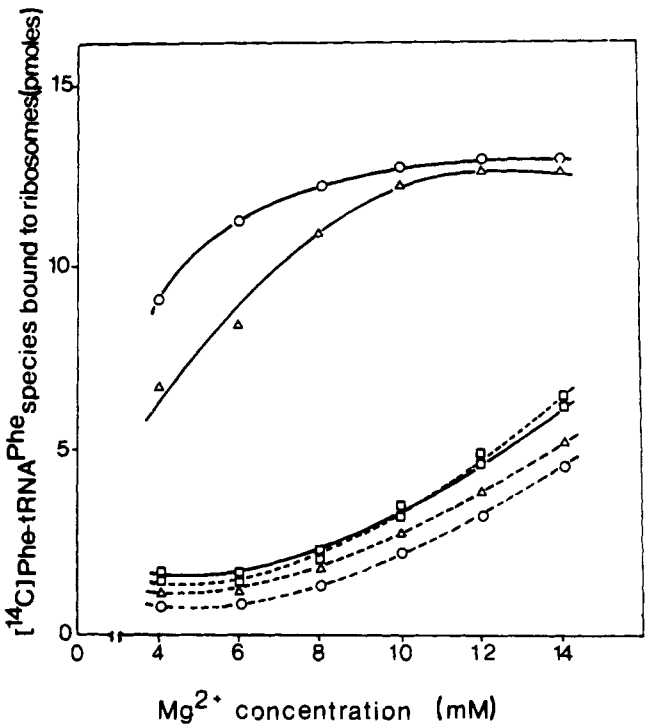

FIGURE 2: Enzymatic and nonenzymatic binding of Phe-tRNA^{Phe} species to ribosomes: dependence on Mg^{2+} concentration. 1.1 A_{260} units of ribosomes precharged with poly(U), 300 pmol of GTP, and 50 pmol of [^{14}C]Phe-tRNA^{Phe}-C-C-A (○), [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA (△), or [^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ (□) (50 Ci/mol) were incubated for 10 min at 0° in the presence (——) or absence (---) of 1.2 μg of EF-T in 75 μl of standard buffer containing $MgCl_2$ at the indicated concentrations. Samples were analyzed by filtration on nitrocellulose filters. Values are corrected for [^{14}C]Phe-tRNA^{Phe} species retained on filters in the absence of ribosomes (0.3–0.4 pmol).

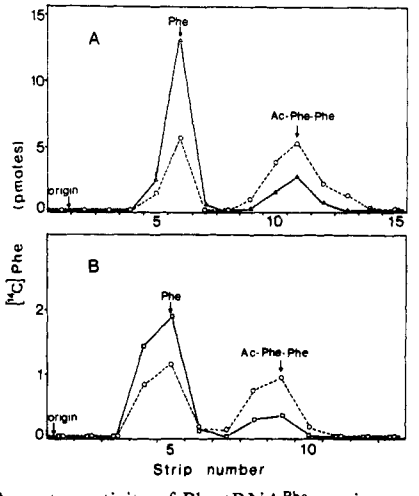

FIGURE 3: Acceptor activity of Phe-tRNA^{Phe} species as measured by Ac-Phe-Phe formation. (A) 180 pmol [^{14}C]Phe-tRNA^{Phe}-C-C-A (○--○) or [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA (△——△) (50 Ci/mol), 4 μg of EF-T, 1 nmol of GTP, and 10 A_{260} units of Ac-Phe-tRNA^{Phe}-poly(U)-ribosome complex (not isolated on sucrose gradient) were incubated for 10 min at 30° in 150 μl of standard buffer with 10 mM $MgCl_2$. Ribosomal complexes (carrying 6 pmol of [^{14}C]phenylalanine/A_{260} unit) were isolated by gel filtration and hydrolyzed with 0.3 M NaOH. Aliquots of the hydrolysates were analyzed by paper chromatography for formation of Ac-Phe-[^{14}C]Phe. (B) 15 pmol of [^{14}C]Phe-tRNA^{Phe}-C-C-A (○---○) or of [^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ (□——□) (513 Ci/mol) and 0.65 A_{260} units of Ac-Phe-tRNA^{Phe}-poly(U)-ribosome complex isolated on a sucrose gradient were incubated 15 min at 30° in 25 μl of standard buffer with 15 mM $MgCl_2$. Ribosomal complexes were isolated by filtration on nitrocellulose filters and hydrolyzed with 0.2 M NH_4OH. Aliquots were analyzed by paper chromatography for formation of Ac-Phe-[^{14}C]Phe.

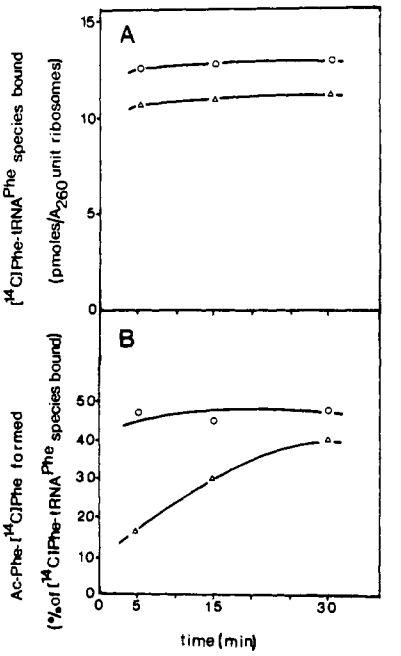

FIGURE 4: Kinetics of enzymatic binding of Phe-tRNA^{Phe}-C-C-3′dA and Phe-tRNA^{Phe}-C-C-A to Ac-Phe-tRNA^{Phe}-poly(U)-ribosome complex and kinetics of peptide-bond formation. 100 pmol of [^{14}C]Phe-tRNA^{Phe}-C-C-A (○) or [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA (△) (513 Ci/mol), 1 nmol of GTP, 5 μg or EF-T, 4 nmol of unlabeled phenylalanine, 40 nmol of ATP, and 5 A_{260} units of isolated Ac-Phe-tRNA^{Phe}-poly(U)-ribosome complex were incubated at 30° in 140 μl of standard buffer with 10 mM $MgCl_2$. At intervals two 20-μl aliquots were withdrawn and filtered separately on nitrocellulose filters. One filter was dried and assayed for [^{14}C]Phe-tRNA^{Phe} species bound to ribosomes (A), while the other was incubated with 0.2 M NH_4OH. The alkaline hydrolysate was analyzed by paper chromatography for dipeptide formation (B). Values were not corrected for [^{14}C]Phe-tRNA^{Phe} species retained on filters in the absence of ribosomes (0.6–0.9 pmol).

ments had been incubated with periodate prior to being aminoacylated in order to completely inactivate possible traces of tRNA^{Phe}-C-C-A in the preparation (Sprinzl *et al.*, 1973).

The kinetics of dipeptide formation showed that the rate of the reaction in the case of the analogs was much slower than with Phe-tRNA^{Phe}-C-C-A. EF-T-directed binding of both [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA and [^{14}C]Phe-tRNA^{Phe} was virtually complete within 5 min at 30° (Figure 4A). At this time maximum formation of Ac-Phe-[^{14}C]Phe was observed in the presence of [^{14}C]Phe-tRNA^{Phe}-C-C-A, while in the case of [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA more than 30 min were required for formation of an equivalent amount of dipeptides (Figure 4B). When Phe-tRNA^{Phe}-C-C-3′dA was bound nonenzymatically to ribosomes, the kinetics of dipeptide formation showed a similar behavior (Figure 5A, B); [^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ reacted in this experimental condition slower than [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA suggesting that opening of the 3′-terminal ribose ring further reduces the ability of tRNA^{Phe} aminoacylated in the 2′-hydroxyl group to function as acceptor. Controls with Ac-Phe-tRNA^{Phe}-C-C-A from yeast confirmed the acceptor activity of modified Phe-tRNA^{Phe} species (not shown).

Donor Activity of Modified Ac-Phe-tRNA^{Phe} Species. The donor activity of the Ac-Phe-tRNA^{Phe} analogs was examined in the puromycin reaction and dipeptide formation with aminoacyl-tRNA bound enzymatically to the A site. *N*-Acetyl derivatives were used because they are better substrates than aminoacyl-tRNA in these reactions (Monro *et al.*, 1968). In the absence (not shown) or presence of EF-G and GTP (Figure 6) modified Ac-Phe-tRNA^{Phe} species did not react with puromycin.

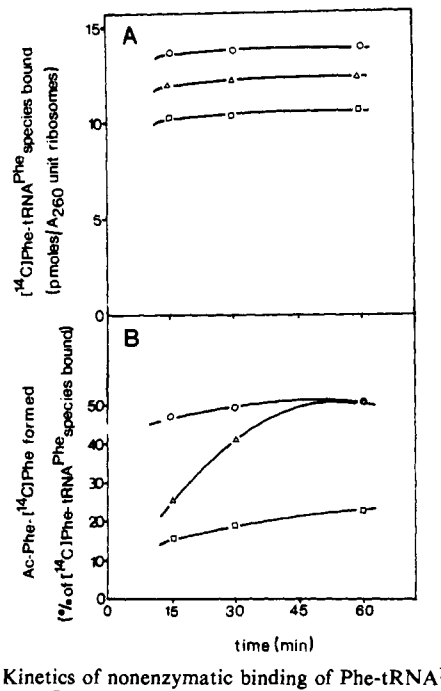

FIGURE 5: Kinetics of nonenzymatic binding of Phe-tRNA^{Phe} species to Ac-Phe-tRNA^{Phe}-poly(U)-ribosome complex and kinetics of peptide-bond formation. 200 pmol of [^{14}C]Phe-tRNA^{Phe}-C-C-A (○), [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA (△), or [^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ (□), respectively (513 Ci/mol), 4 nmol of unlabeled phenylalanine, 40 nmol of ATP, and 5 A_{260} units of Ac-Phe-tRNA^{Phe}-poly(U)-ribosome complex were incubated at 30° in 140 μl of standard buffer with 15 mM $MgCl_2$. [^{14}C]Phe-tRNA^{Phe} species bound to ribosomes and formation of Ac-Phe-[^{14}C]Phe were determined as indicated in the legend of Figure 4.

Likewise, chromatograms of alkaline hydrolysates of ribosomal complexes carrying modified Ac-[^{14}C]Phe-tRNA^{Phe} species did not show formation of Ac-[^{14}C]Phe-Phe when Phe-tRNA^{Phe} from *E. coli* was enzymatically bound to the A site (Figure 7). The lack of donor activity of Ac-Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ was previously reported by Hussain and Ofengand (1973).

The Ac-Phe-tRNA^{Phe} analogs do bind to the ribosomal P site as shown in the series of experiments illustrated in Table II. Poly(U)-ribosomes were first incubated for 10 min at 30° with either tRNA^{Phe}-C-C-A, as control, or with native or modified Ac-[^{14}C]Phe-tRNA^{Phe}. At the end of this incubation binding of Ac-[^{14}C]Phe-tRNA^{Phe} species to ribosomes was determined in an aliquot of the reaction mixture. Two additional aliquots were further incubated for 5 min at 30° in the presence of [^{3}H]Phe-tRNA^{Phe}-C-C-A with or without EF-T and GTP, and then binding of the [^{3}H]Phe-tRNA^{Phe}-C-C-A and Ac-[^{14}C]Phe-tRNA^{Phe} species to ribosomes was determined. It was observed that like non-aminoacylated tRNA^{Phe}, native and modified Ac-Phe-tRNA^{Phe} species do not prevent the enzymatic binding of [^{3}H]Phe-tRNA^{Phe}-C-C-A to the A site and, in turn, the binding of [^{3}H]Phe-tRNA^{Phe}-C-C-A promotes little displacement (20–25%) or Ac-[^{14}C]Phe-tRNA^{Phe} species already bound to ribosomes prior to the second incubation. These results, together with the observation that under these experimental conditions Ac-Phe-tRNA^{Phe}-C-C-A functions as donor (Figure 7), clearly indicate that modified Ac-Phe-tRNA^{Phe} species bind to the ribosomal P site like Ac-Phe-tRNA^{Phe}-C-C-A. Ability of Phe-tRNA-C-C-A$_{oxi\text{-}red}$ to bind to the P site was already suggested by Ofengand and Chen (1972).

We examined the products formed in the in vitro system for poly(phenylalanine) synthesis when [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA or 1^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ was present instead of [^{14}C]Phe-tRNA^{Phe}-C-C-A. Only [^{14}C]Phe was detected,

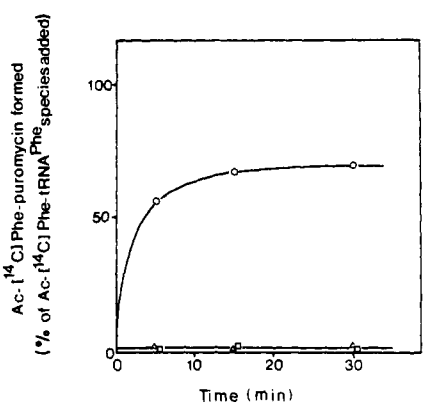

FIGURE 6: Donor activity of Ac-Phe-tRNA^{Phe} species as measured by the puromycin reaction. 10 A_{260} units of ribosomes precharged with poly(U) and 140 pmol of Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-A (○), Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-3′dA (△), or Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ (□) (50 Ci/mol) were incubated 10 min at 30° in 100 μl of standard buffer with 10 mM $MgCl_2$. 5 μl of standard buffer with 10 mM $MgCl_2$ containing 100 nmol of puromycin, 1 μg of EF-G, and 10 nmol of GTP were then added and incubation at 30° was continued. Controls were run in parallel in the absence of puromycin. At intervals 30-μl aliquots were withdrawn and assayed for formation of acid-soluble Ac-[^{14}C]Phe-puromycin.

thus confirming that the modified tRNA^{Phe} species cannot function both as acceptor and donor.

Modified Ac-Phe-tRNA^{Phe} Species Block P Site. Incubation of poly(U)-ribosomes with Ac-Phe-tRNA^{Phe}-C-C-3′dA or Ac-Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ in the presence of 10 mM $MgCl_2$ inhibited poly(phenylalanine) synthesis subsequently carried out in the presence of Phe-tRNA^{Phe}-C-C-A (Figure 8A). The fact that both modified Ac-Phe-tRNA^{Phe} species did not affect the initial rate of phenylalanine incorporation when added simultaneously with Phe-tRNA^{Phe}-C-C-A (Figure 8A, dashed line) indicated that this inhibition requires prior binding of the modified Ac-Pte-tRNA^{Phe} species to ribosomes. Furthermore the continuous inhibition during poly(phenylalanine) synthesis suggests that binding of modified Ac-Phe-tRNA^{Phe} species to ribosomes is rather stable; after 20-min incubation, only about 25% of the bound analogs had been released from ribosomes (Figure 8B). Since Ac-Phe-tRNA^{Phe} analogs do not function as donor (Figures 6 and 7) these results show that they inhibit the entrance of native Phe-tRNA^{Phe} into the P site which is required for initiation of poly(phenylalanine) synthesis.

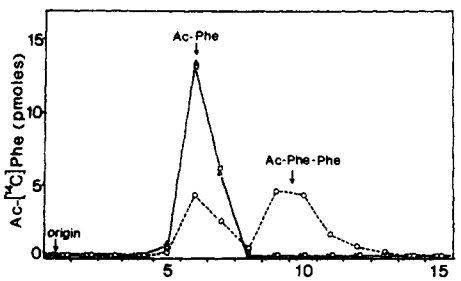

FIGURE 7: Donor activity of Ac-Phe-tRNA^{Phe} species as measured by Ac-Phe-Phe formation. 10 A_{260} units of ribosomes precharged with poly(U) and 120 pmol of Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-A (○---○), Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-3′dA (△——△), or Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ (□——□) (50 Ci/mol) were incubated 10 min at 30° in 100 μl of standard buffer with 10 mM $MgCl_2$. 50 μl of standard buffer with 10 mM $MgCl_2$ containing 400 pmol of unlabeled Phe-tRNA^{Phe}-C-C-A from *E. coli*, 5 μg of EF-T, and 1.4 nmol of GTP were added to each reaction mixture and incubation was further continued for 10 min at 30°. Ribosomal complexes (containing 6–7 pmol of Ac-[^{14}C]Phe/A_{260} unit) were isolated by gel filtration on Sepharose 4B, hydrolyzed with 0.3 M NaOH and analyzed by paper chromatography.

TABLE II: EF-T-Directed Binding of Phe-tRNA^{Phe}–C–C–A to Ribosomes Carrying Ac-Phe-tRNA^{Phe} Species.[a]

First Incubn Addns	Second Incubn Addns	Aminoacylated tRNA^{Phe} Bound to Ribosomes (pmol/A_{260} Unit of Ribosomes)	
		Ac-[^{14}C]Phe-tRNA^{Phe} Species	[^{3}H]Phe-tRNA^{Phe}–C–C–A
tRNA^{Phe}–C–C–A	[^{3}H]Phe-tRNA^{Phe}–C–C–A		1.6
tRNA^{Phe}–C–C–A	[^{3}H]Phe-tRNA^{Phe}–C–C–A + EF-T + GTP		14.6
Ac-[^{14}C]Phe-tRNA^{Phe}–C–C–A	No incubation	12.1	
Ac-[^{14}C]Phe-tRNA^{Phe}–C–C–A	[^{3}H]Phe-tRNA^{Phe}–C–C–A	10.3	1.1
Ac-[^{14}C]Phe-tRNA^{Phe}–C–C–A	[^{3}H]Phe-tRNA^{Phe}–C–C–A + EF-T + GTP	10.2	15.1
Ac-[^{14}C]Phe-tRNA^{Phe}–C–C–3′dA	No incubation	10.2	
Ac-[^{14}C]Phe-tRNA^{Phe}–C–C–3′dA	[^{3}H]Phe-tRNA^{Phe}–C–C–A	8.4	1.2
Ac-[^{14}C]Phe-tRNA^{Phe}–C–C–3′dA	[^{3}H]Phe-tRNA^{Phe}–C–C–A + EF-T + GTP	8.4	15.4
Ac-[^{14}C]Phe-tRNA^{Phe}–C–C–A$_{oxi\text{-}red}$	No incubation	10.2	
Ac-[^{14}C]Phe-tRNA^{Phe}–C–C–A$_{oxi\text{-}red}$	[^{3}H]Phe-tRNA^{Phe}–C–C–A	8.0	1.4
Ac-[^{14}C]Phe-tRNA^{Phe}–C–C–A$_{oxi\text{-}red}$	[^{3}H]Phe-tRNA^{Phe}–C–C–A + EF-T + GTP	7.8	15.5

[a] First incubation: 1.4 A_{260} units of poly(U)–ribosomes were incubated 10 min at 30° with 35 pmol of Ac-Phe-tRNA^{Phe} species (513 Ci/mol) in 70 μl of standard buffer with 10 mM $MgCl_2$. A 20-μl aliquot of each reaction mixture was immediately assayed for Ac-[^{14}C]Phe-tRNA^{Phe} species bound to ribosomes. Second incubation: two additional 20-μl aliquots were further incubated 5 min at 30° in 15 μl of standard buffer with 10 mM $MgCl_2$ containing 25 pmol of [^{3}H]Phe-tRNA^{Phe}–C–C–A (20,000 Ci/mol) in the presence or absence of 1 μg or EF-T and 0.5 nmol of GTP. After incubation samples were assayed for Ac-[^{14}C]Phe-tRNA^{Phe} species and [^{3}H]Phe-tRNA^{Phe}–C–C–A bound to ribosomes. Controls were run in parallel by adding in the first incubation 50 pmol of non-aminoacylated yeast tRNA^{Phe} instead of the Ac-[^{14}C]Phe-tRNA^{Phe} species.

Inhibition of Peptide-Chain Elongation. The effect of Phe-tRNA^{Phe}-C-C-3′dA and of Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ on peptide-chain elongation is illustrated in Figure 9. In these experiments poly(phenylalanine) synthesis was first allowed to take place for 10 min at 30° in the presence of a saturating amount of tRNA^{Phe}-C-C-A (dashed line) and under conditions in

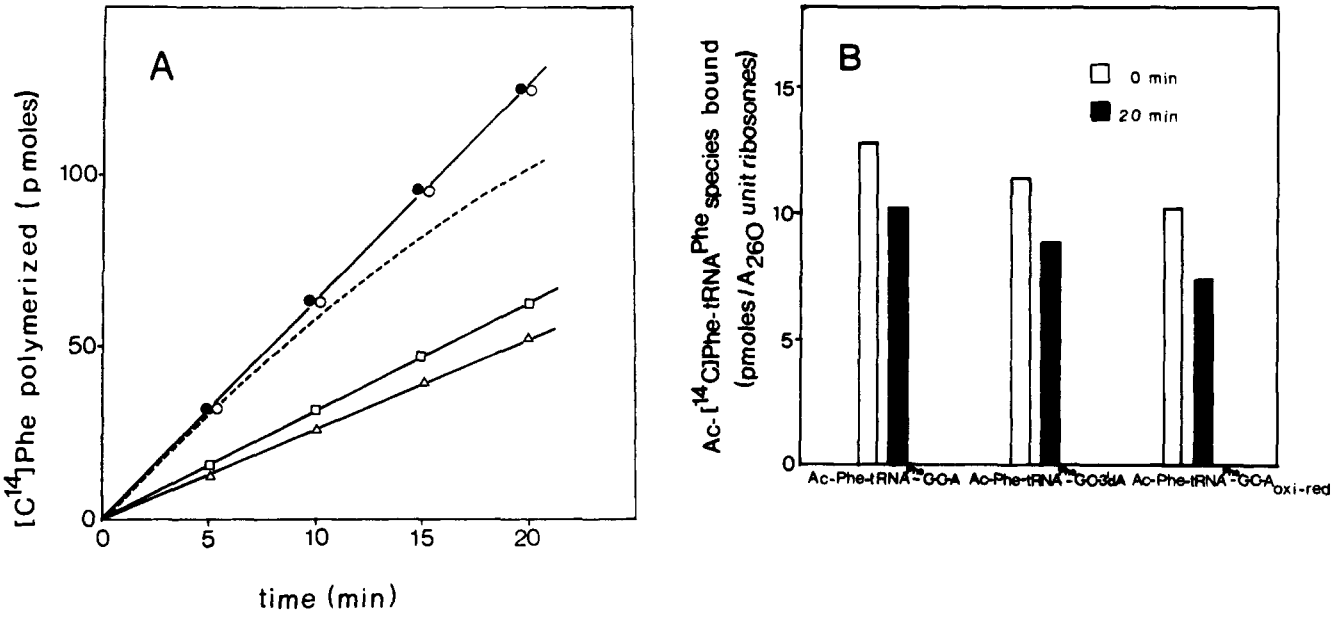

FIGURE 8: Effect of Ac-Phe-tRNA^{Phe} species on polypeptide chain initiation. (A) First incubation: 1.2 A_{260} units of poly(U)–ribosomes and 30 pmol of Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-A (○), Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-3′dA (△), or Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ (□) (513 Ci/mol) were incubated 10 min at 30° in 40 μl of standard buffer with 10 mM $MgCl_2$ and then chilled to 0°. A control was run in parallel omitting Ac-Phe-tRNA^{Phe} species (●). Second incubation: to each reaction mixture 50 μl of standard buffer with 10 mM $MgCl_2$ containing 26 nmol of GTP, 50 nmol of ATP, 0.4 nmol of [^{14}C]phenylalanine (513 Ci/mol), 0.12 nmol of yeast tRNA^{Phe}-C-C-A, 1 μg of yeast phenylalanyl-tRNA synthetase, 0.12 μg of EF-G, and 2.5 μg of EF-T were added and samples were then incubated at 30°. At 5-min intervals 20-μl aliquots were withdrawn and assayed for [^{14}C]phenylalanine incorporated into hot trichloroacetic acid insoluble material. The dashed line represents the kinetics of [^{14}C]phenylalanine incorporation determined when the first incubation was omitted and Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-3′dA or Ac-[^{14}C]Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ was added to reaction mixtures together with the other components just before the second incubation. Values on abscissa refer to the total volume of the reaction mixture (90 μl). (B) Under the same experimental conditions as in (A) except that unlabeled phenylalanine replaced [^{14}C]phenylalanine in the second incubation; samples were analyzed for Ac-[^{14}C]Phe-tRNA^{Phe} species bound to ribosomes after the first (open column) and second (full column) incubation at 30° by filtration on nitrocellulose filters.

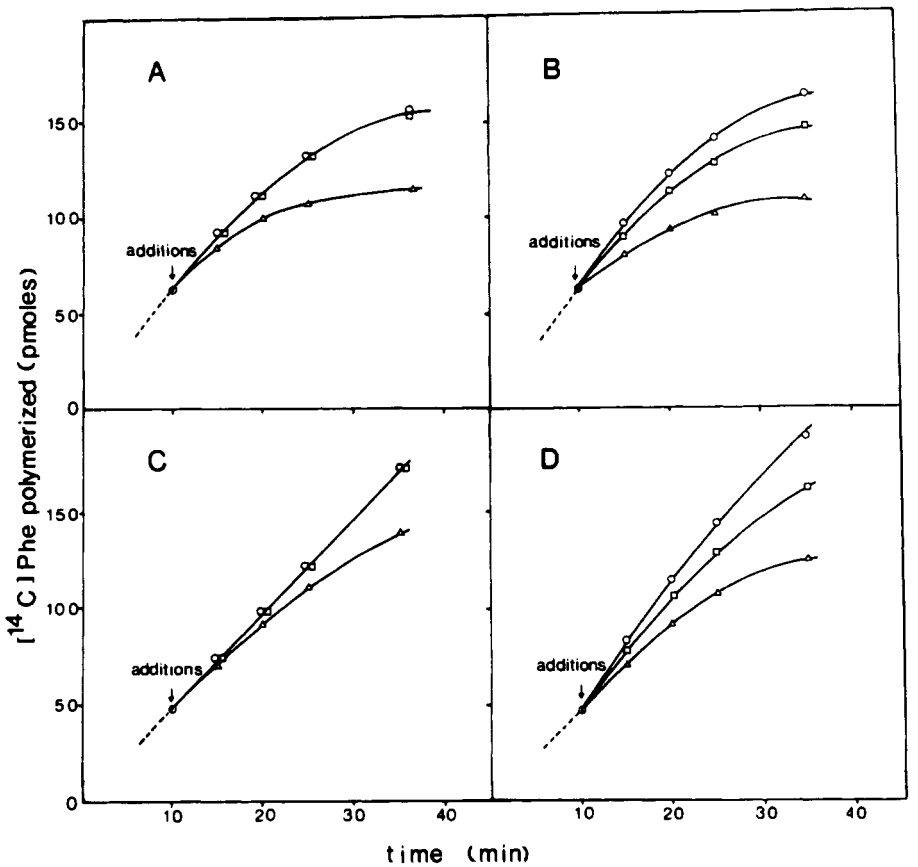

FIGURE 9: Effect of Phe-tRNA^{Phe} species on polypeptide chain elongation. Reaction mixture containing 12 A_{260} units of ribosomes precharged with poly(U), 400 nmol of GTP, 700 nmol of ATP, 5 nmol of [^{14}C]phenylalanine (513 Ci/mol), 0.9 nmol of yeast tRNA^{Phe}-C-C-A, 5 μg of yeast phenylalanyl-tRNA synthetase, and either 7.5 μg of EF-G and 6 μg of EF-T (A and B), or 2.5 μg of EF-G and 45 μg of EF-T (C and D) were incubated 10 min at 30° in 660 μl of standard buffer with 6 mM $MgCl_2$ and then chilled to 0° in ice–water bath. A 25-μl aliquot was withdrawn to determine [^{14}C]phenylalanine incorporation at this time (⊙). Reaction mixtures were then divided into six 100-μl aliquots, three of which received 10 μl of standard buffer with 6 mM $MgCl_2$, without (○) or with 240 pmol of tRNA^{Phe}-C-C-3′dA (△) or tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ (□) (A and C). To the other three aliquots the same components were added except that $MgCl_2$ in the standard buffer was 50 mM in order to increase final Mg^{2+} concentration to 10 mM (B and D). Incubation at 30° was resumed and, at intervals, 25-μl aliquots were withdrawn and assayed for [^{14}C]phenylalanine incorporation into hot trichloroacetic acid insoluble material. Values on abscissa refer to the total volume of the reaction mixture (110 μl).

which either EF-T (Figures 9A, B) or EF-G (Figures 9C, D) was rate limiting. Reaction mixtures were chilled to 0° and, after addition of modified tRNA^{Phe}, poly(phenylalanine) synthesis was resumed at 30° in the presence of either 6 mM Mg^{2+} (Figures 9A, C) or 10 mM Mg^{2+} (Figures 9B, D). At the lower Mg^{2+} concentration only Phe-tRNA^{Phe}-C-C-3′dA was inhibitory (Figures 9A, C). Because the nonenzymatic binding of Phe-tRNA^{Phe} to the A site is negligible at 6 mM Mg^{2+}, this inhibition represents a competitive interaction of Phe-tRNA-C-C-3′dA with EF-T-directed binding of Phe-tRNA^{Phe}-C-C-A. Phe-tRNA^{Phe}-C-C-A$_{oxi\text{-}red}$ showed inhibitory activity only at 10 mM Mg^{2+}, *i.e.*, when the Mg^{2+} concentration was sufficiently high to allow some nonenzymatic binding to the ribosomal A site (Figures 9B, D).

Phe-tRNA^{Phe}-C-C-3′dA bound enzymatically to ribosomes seems not to irreversibly block the A site. If this were so, its inhibitory activity should depend only on the ratio of Phe-tRNA^{Phe}-C-C-3′dA to Phe-tRNA^{Phe}-C-C-A and on the rate of poly(phenylalanine) synthesis. The observation that Phe-tRNA^{Phe}-C-C-3′dA was consistently more inhibitory when EF-T was rate limiting (compare Figure 9A with C and Figure 9B with D) indicates that Phe-tRNA^{Phe}-C-C-A can displace Phe-tRNA^{Phe}-C-C-3′dA from the ribosomal A site. This was confirmed by showing that [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA bound enzymatically to the A site was extensively removed from ribosomes by unlabeled Phe-tRNA^{Phe}-C-C-A in the presence of EF-T, while [^{14}C]Phe-tRNA^{Phe}-C-C-A did not exchange under the same conditions (Figure 10).

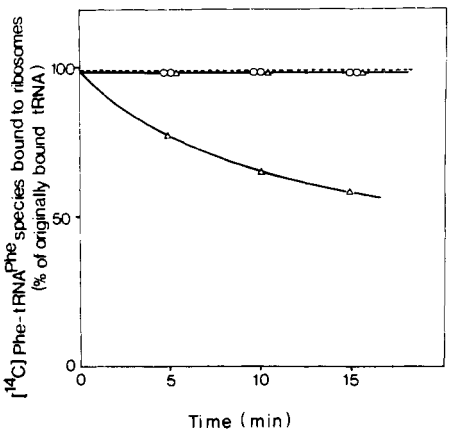

FIGURE 10: Displacement of Phe-tRNA^{Phe}-C-C-3′dA bound enzymatically to the A site by free Phe-tRNA^{Phe}-C-C-A. 3 A_{260} units of ribosomes, 12 μg of poly(U), and 120 pmol of tRNA^{Phe}-C-C-A were incubated 10 min at 30° in 60 μl of standard buffer with 10 mM $MgCl_2$. Then 70 μl of standard buffer with 10 mM $MgCl_2$ containing 8 μg of EF-T, 0.5 nmol of GTP, and 65 pmol of [^{14}C]Phe-tRNA^{Phe}-C-C-A (○), or [^{14}C]Phe-tRNA^{Phe}-C-C-3′dA (△) were added. Reaction mixtures were incubated for 5 min at 30° then chilled to 0°. Binding of [^{14}C]Phe-tRNA^{Phe} species to ribosomes at this time was determined with 15-μl aliquots Two 50-μl aliquots of the reaction mixtures were further incubated at 30° after addition of 5 μl of standard buffer with 10 mM $MgCl_2$ without (---) or with (——) 120 pmol of unlabeled yeast Phe-tRNA^{Phe}-C-C-A. At intervals 15-μl aliquots were withdrawn and analyzed for [^{14}C]Phe-tRNA^{Phe} species bound to ribosome.

Discussion

The structural requirements for the recognition of aminoacyl-tRNAs by EF-Tu have been investigated in a number of laboratories. Selective modifications on the anticodon loop (Krauskopf *et al.*, 1972; Chen *et al.*, 1970; Ghosh and Ghosh, 1970; Thang *et al.*, 1971), anticodon stem (Abelson *et al.*, 1970), dihydrouridine loop (Abelson *et al.*, 1970), or stem (Krauskopf *et al.*, 1972) region do not affect recognition by EF-Tu. On the other hand, the enzymatic binding of aminoacylated tRNA to ribosomes is severely affected by modification of the terminal adenosine. Chen and Ofengand (1970) and Ofengand and Chen (1972) have shown that Phe-tRNA^{Phe}-C-C-$A_{oxi\text{-}red}$ is not able to form a ternary complex with EF-Tu and GTP and does not bind enzymatically to the ribosomal A site. These authors suggested that the lack of functional activity of this tRNA might be explained either by a stereochemical requirement for the 2′,3′-carbon bond or by the necessity of an amino acid transfer from the 2′ to the 3′ position. Our results show that in contrast to Phe-tRNA^{Phe}-C-C-$A_{oxi\text{-}red}$, Phe-tRNA^{Phe}-C-C-3′dA is bound enzymatically to ribosomes and competes with Phe-tRNA^{Phe}-C-C-A in this reaction. Transacylation from the 2′- to 3′-hydroxyl group and even the presence of the 3′-OH group are therefore not required for EF-Tu recognition. The possibility remains that either EF-Tu does not discriminate between the 2′ and 3′ isomer of aminoacyl-tRNA or that the 2′ isomer is the only active species. The lack of functional activity of Phe-tRNA^{Phe}-C-C-$A_{oxi\text{-}red}$ in this reaction only can be due to the stereochemical consequences of the absence of the 2′,3′-carbon bond on the terminal ribose. Recent spectroscopic investigations on tRNAs carrying the fluorescent formycin residue in the 3′-terminal position (tRNA^{Phe}-C-C-F and tRNA^{Phe}-C-C-$F_{oxi\text{-}red}$) show that an intact 3′-terminal ribose ring stabilizes the stacking interactions between the bases of 3′-terminal nucleotides (Maelicke *et al.*, 1974). A defined structure of the -C-C-A end, which involves these stacking interactions, seems to be responsible for the high specificity of the aminoacyl-tRNA recognition by EF-Tu. Results obtained by chemical modifications of formylmethionyl-tRNA in which a new base pair was introduced at the end of the acceptor stem point in the same direction (Schulman and Her, 1973). The presence of the new base pair in this tRNA, which is normally not recognized by EF-Tu (Ono *et al.*, 1968), led to structural stabilization of the -C-C-A end and to recognition by EF-Tu. Binding of Phe-tRNA^{Phe}-C-C-3′dA to the ribosomal A site in the presence of EF-T and GTP is, however, not as tight as that of Phe-tRNA^{Phe}-C-C-A as shown by the higher Mg^{2+} requirement and by its displacement from the A-site by free Phe-tRNA^{Phe}-C-C-A. This suggests alterated interaction between Phe-tRNA^{Phe}-C-C-3′dA and the peptidyltransferase center since only the 3′-terminal ribose which is directly involved in this interaction is modified. This conclusion is supported by the observation that also Phe-tRNA^{Phe}-C-C-A shows a similar behavior when bound enzymatically to the A site in the presence of 5′-guanylylmethylenediphosphonate, a nonhydrolyzable analog of GTP. In this case stability of the Phe-tRNA–ribosome complex is greatly decreased (Hamburger *et al.*, 1973) and the bound Phe-tRNA^{Phe}-C-C-A becomes readily exchangeable with free Phe-tRNA^{Phe}-C-C-A (Chinali and Parmeggiani, unpublished results) because the absence of GTP hydrolysis prevents the release of EF-Tu from ribosomes (Lucas-Lenard *et al.*, 1969; Skoultchi *et al.*, 1969), and so blocks functional interaction of the aminoacyl-tRNA with the peptidyltransferase center (Haenni and Lucas-Lenard, 1968). The weaker binding of Phe-tRNA^{Phe}-C-C-3′dA to the A site in spite of GTP hydrolysis and EF-Tu release is interpreted as due to a less effective interaction of this tRNA with the peptidyltransferase center.

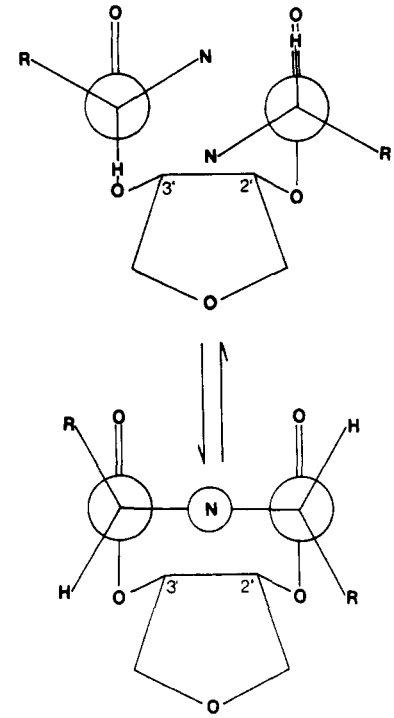

FIGURE 11: Above: stereochemically favored conformations of the aminoacyl residues bound to the 2′ or 3′ position of the terminal ribose of tRNA. Below: torsion of C–C bond leading to a conformation where the amino groups of both analogs occupy an identical position.

The 2′ isomer of puromycin (Nathans and Neidel, 1963), adenosine analogs aminoacylated on the 2′-OH group (Chládek *et al.*, 1973), and the C-A-C-C-$A_{oxi\text{-}red}$-Phe fragment isolated from enzymatically aminoacylated tRNA^{Phe}-C-C-$A_{oxi\text{-}red}$ (Hussain and Ofengand, 1973) do not react with peptidyl-tRNA. Consequently it has been assumed that only the 3′ isomers of aminoacyl-tRNA can function as acceptor of the peptidyl residue. The results presented here show that Phe-tRNA^{Phe}-C-C-3′dA and to a lesser extent Phe-tRNA^{Phe}-C-C-$A_{oxi\text{-}red}$ react with Ac-Phe-tRNA^{Phe}-C-C-A bound to the P site of the ribosomes. Thus, the corresponding dipeptide is formed even if the amino acid is bound to the 2′-hydroxyl group. The presence of the vicinal hydroxyl group and of the intact ribose ring albeit not essential seem to greatly influence the rate of peptide-bond formation. The slower reactivity of these modified Phe-tRNA^{Phe} species may be a consequence of a weak binding to the peptidyltransferase center. From the fact that peptide formation occurs, it can be concluded that the 2′-aminoacylated, modified tRNA^{Phe} species still have a correct orientation of the -C-C-A terminus and of the amino group of the attached amino acid. The required stereochemical arrangement is probably stabilized by interactions between other regions of the tRNA molecule and the ribosome. The absence of such stabilizing interactions is presumably one reason responsible for the inactivity of the 2′ isomer of puromycin and of C-A-C-C-$A_{oxi\text{-}red}$-Phe fragment. The fact that the 3′ isomer is the active form of puromycin together with the observation that Phe-tRNA^{Phe}-C-C-3′dA interacts weakly with the A site indicate, however, that the 3′ isomer of aminoacyl-tRNA should be more effective in binding to the peptidyltransferase center and for reacting with peptidyl-tRNA.

Recently Fraser and Rich (1973) have shown that Phe-tRNA^{Phe}-C-C-3′amino-3′deoxy-A carrying phenylalanine bound to the 3′ position by an amide linkage does, like puromy-

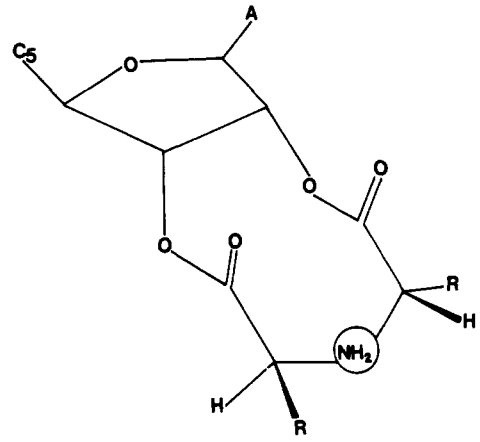

FIGURE 12: Schematic drawing of the stereochemical arrangement illustrated in Figure 11, below.

cin, act as an acceptor for the Ac-Phe residue. Therefore both 2′ and 3′ isomers of aminoacyl-tRNA are able to accept the peptidyl residue. Given the reasonable assumption that a defined and unique position of the α-amino group of the accepting amino acid is required for peptide-bond formation, one has to postulate that the amino groups in both 2′- and 3′-acylated tRNA have to occupy an identical spatial position. In fact, there is only one possible conformation in which both 2′- and 3′-aminoacyl-tRNA isomers have the amino group in an identical position. Endo conformations of the carbonyl groups, in which the carbonyl oxygen points beneath the ribose ring, are excluded by the results of X-ray (Sundaralingam and Arora, 1969) and conformation analysis (Yathindra and Sundaralingam, 1973) of puromycin and acetyl nucleosides (Mathieson, 1965; Saenger and Suck, 1971). Taking these data into account, the sterically most favoured conformation for the aminoacyl residue of 2′-aminoacyl-tRNA or 3′-aminoacyl-tRNA is shown in Figure 11 (above). A slight distortion effected either by conformational changes in the ribose ring or in the amino acid brings the amino groups to an identical position, as indicated in Figure 11 (below)and depicted in Figure 12.

Ac-Phe-tRNA^{Phe}-C-C-3′dA and Ac-Phe-tRNA^{Phe}-C-C-$A_{oxi\text{-}red}$ appear to be completely devoid of donor activity, despite the fact that they are bound to the ribosomal P site almost as tightly as Ac-Phe-tRNA^{Phe}-C-C-A. Thus, either the 2′ isomer of aminoacyl-tRNA is not the active species or the presence of the intact terminal ribose ring with a free vicinal hydroxyl group is required for the release of the peptidyl residue. Also the Ac-Phe-tRNA^{Phe}-C-C-3′amino-3′deoxy-A is inactive as donor (Fraser and Rich, 1973). This can, however, be ascribed to the inability of the peptidyltransferase to break the amide linkage. At present, it cannot be decided which of the two isomers of peptidyl-tRNA is preferentially used in the donor reaction. The chemical aminocylation of tRNA^{Phe}-C-C-2′dA should represent the proper tool for answering this question.

In conclusion, the activity of aminoacyl-tRNA in protein synthesis shows the following structural requirement for the 3′ terminus. EF-Tu-dependent binding and GTP hydrolysis require an intact ribose ring, but no vicinal hydroxyl group; the amino acid position is either unessential or only the 2′ isomer is used. Both 2′ and 3′ isomers can accept the peptidyl residue; the presence of vicinal hydroxyl group or of intact ribose ring is not essential, but greatly influences the rate of peptide-bond formation. Ac-Phe-tRNA^{Phe}-C-C-3′dA is unable to donate the peptidyl residue either because the vicinal hydroxyl group is necessary or transacylation to the 3′ position is required.

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Ribonucleic Acid Dependent Deoxyribonucleic Acid Synthesis by *Escherichia coli* Deoxyribonucleic Acid Polymerase I. Characterization of the Polymerization Reaction†

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ABSTRACT: The RNA dependent DNA polymerization activity of *Escherichia coli* polymerase I is an intrinsic part of the enzyme molecule as shown by several methods (gel filtration, isoelectric focusing) and a requirement for enzyme-bound Zn^{2+}. The DNA product of the reaction is covalently bound to the RNA initiator-template complex. The stoichiometry between the enzyme and the RNA template has been investigated and the mole ratio of the two was found to be critical for extended DNA synthesis and for the ultimate size of the product. The *"de novo"* poly[d(A-T)]·[d(A-T)] synthesis which occurs concurrently with the RNA dependent DNA reaction was minimized by using a high concentration of Mg^{2+} (8 mM) in the reaction mixture. Our evidence indicates that the polymerase has a double-stranded RNA endonuclease activity which produces 3′-OH termini; this enables the polymerase to create additional points of initiation for DNA synthesis on RNA molecules containing double-stranded regions.

Now that it is possible to obtain DNA copies of unique sequences of RNA, new and powerful methods are available for the analysis of viral replication, integration, and function as well as for the investigation of RNA metabolism in cellular differentiation. These DNA copies have been synthesized from RNA templates using RNA dependent DNA polymerases ("reverse transcriptases") from animal tumor viruses, particularly avian myeloblastosis virus, AMV (Temin and Baltimore, 1972). We and others have shown that natural RNAs can also be faithfully copied by *Escherichia coli* DNA polymerase I (Pol I)[1] (Loeb *et al.*, 1973; Modak *et al.*, 1973). This finding has recently been confirmed by Gulati *et al.* (1974) who found that the DNA copies of rabbit globin mRNA synthesized by Pol I were indistinguishable by hybridization kinetics from those synthesized by AMV polymerase. Highly purified Pol I is available in large quantities and appears to be an ideal tool for copying a variety of RNA templates. The DNA copies can then be isolated and used as templates for repetitive net synthesis. This procedure could provide large amounts of specific complementary DNAs for the study of the role of a particular RNA in cellular metabolism.

It should be noted that a comparison of the specific activities of purified Pol I and the DNA polymerases from tumor viruses indicates that they are all more active in copying DNA templates than RNA templates (Loeb, 1974). The efficiency of Pol I in copying RNA templates is similar to that of the "reverse transcriptases." However, Pol I copies "activated" DNA 500–1000 times more efficiently than rRNA (Loeb *et al.*, 1973).

Hitherto, even though most of the sequences in the RNA template appear to be copied with viral RNA (Garapin *et al.*, 1973; Tavitian *et al.*, 1974), the DNA copies have been small molecules compared to the molecular size of the RNA template no matter whether the polymerase used is Pol I or a "reverse transcriptase" (Gulati *et al.*, 1974).

To better understand how this is possible, we have analyzed in detail the RNA dependent DNA polymerization reaction using Pol I. To do this, we have used 28S rRNA as a template since, unlike most other RNAs investigated, no added oligonucleotides, which might make the interpretation of the reaction more complex, were necessary to initiate the polymerization reaction. We have already shown in a previous paper (Loeb *et al.*, 1973) that the requirements for catalysis of polymerization using Pol I with an RNA template are essentially identical with those using Pol I with a DNA template.

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[1] Abbreviation used is: Pol I, *E. coli* DNA polymerase I.