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Enzymatic Acylation of Oxidized–Reduced Transfer Ribonucleic Acid by *Escherichia coli,* Yeast, and Rat Liver Synthetases Occurs Almost Exclusively at the 2′-Hydroxyl†

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ABSTRACT: The position of acylation in seven tRNA synthetase systems was examined. Enzymatic acylation with phenylalanine of the nonisomerizable tRNA analog, oxidized–reduced tRNA, made it possible to isolate a stable 3′-terminal fragment. Reaction with *N*-benzyloxycarbonyl chloride, followed by RNase hydrolysis, yielded *N*-benzyloxycarbonylphenylalanyladenosine$^{ox\text{-}red}$ which was separated into its 2′ and 3′ isomers by thin-layer chromatography. When yeast synthetase attached phenylalanine to yeast tRNA^{Phe} under normal conditions, or to *E. coli* tRNA^{Phe} or *E. coli* tRNA^{Val} in the presence of 20% dimethyl sulfoxide, 98–99% of the product was the 2′ isomer. When *Escherichia coli* synthetase acylated *E. coli* tRNA^{Phe} normally, or yeast tRNA^{Phe} in the presence of 1.5 M ammonium sulfate, 95–97% of the product was the 2′ isomer. Acylation of rat liver tRNA^{Phe} or yeast tRNA^{Phe} by rat liver synthetase gave 90–92% 2′ isomer. The results with the yeast synthetase–yeast tRNA^{Phe} system were verified by nuclear magnetic resonance analysis which showed that >85% of the product was the 2′ isomer. Retention of a high degree of specificity for the 2′-hydroxyl under such widely varying conditions leads to the conclusion that this is the normal site of acylation in the biosynthesis of aminoacyl tRNA.

In a previous publication from this laboratory, the activity of Phe-tRNA$^{ox\text{-}red}$ [1] prepared by enzymatic acylation of oxidized–reduced yeast tRNA^{Phe} was examined in several intermediate reactions of protein synthesis (Ofengand and Chen, 1972). This modification was found to block EFTu-GTP recognition, binding to the A site on the ribosomes, and to diminish its affinity for nonenzymatic binding at the ribosomal P site. At that time, we suggested the possibility that the reason for this lack of activity lay not in the cleavage of the $C_{2'}$–$C_{3'}$ bond but was due to enzymatic acylation of the 2′-hydroxyl, failure to isomerize due to the lack of vicinal hydroxyl groups in the opened ribose ring, and a specificity for 3′-esterified AA-tRNA in protein synthesis.

Subsequent work (Hussain and Ofengand, 1973) confirmed this hypothesis by the demonstration that chemically synthesized Ado$^{ox\text{-}red}$(Phe) [IV, Figure 1], a mixture of the 2′ and 3′ isomers, was active as a peptidyl transferase acceptor while biologically synthesized Ado$^{ox\text{-}red}$(Phe) and CACCA$^{ox\text{-}red}$(Phe), prepared from enzymatically acylated tRNA$^{ox\text{-}red}$, were completely inactive. Since puromycin, a 3′-analog of aminoacyl-tRNA, is the classic acceptor for peptidyl transferase, it was concluded (a) that peptidyl transferase was 3′ specific, (b) that enzymatic acylation was 2′ specific, and (c) that isomerization between 3′ and 2′ does not occur in the open ring ester system.

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[1] Abbreviations used are: tRNA$^{ox\text{-}red}$, tRNA which has been first oxidized at the 3′-terminal end with periodate, and then reduced to the diol by $NaBH_4$; Ado$^{ox\text{-}red}$, adenosine modified as above, the correct nomenclature is 2-(adenin-9-yl)-4′-methylol-3-oxopentane-1,5-diol (see Figure 1, $R_1 = R_2 = R_3 = H$); EFTu, one of the peptide chain elongation factors from *E. coli;* Z, *N*-benzyloxycarbonyl; MeOTr, *p*-methoxytrityl; A_{260}, that amount of tRNA in 1 ml which has an absorbance at 260 nm of 1.0 in a 1-cm path cell; Ado(Z-Phe), a mixture of Ado(2′-Z-Phe) and Ado(3′-Z-Phe); Ado(Phe), a mixture of Ado(2′-Phe) and Ado(3′-Phe).

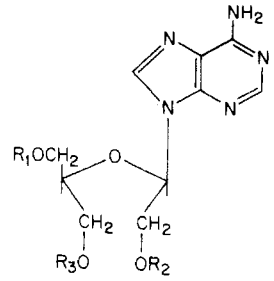

FIGURE 1: Structure of oxidized-reduced adenosine derivatives. (I) $Ado^{ox\text{-}red}(2'\text{-Z-Phe})$; $R_1 = R_3 = H$; R_2 = Z-Phe; (II) $Ado^{ox\text{-}red}(3'\text{-Z-Phe})$; $R_1 = R_2 = H$; R_3 = Z-Phe); (III) MeOTr-$Ado^{ox\text{-}red}$; $R_2 = R_3 = H$; R_1 = MeOTr; (IVa) $Ado^{ox\text{-}red}(3'\text{-Phe})$; $R_1 = R_2 = H$; R_3 = Phe; (IVb) $Ado^{ox\text{-}red}(2'\text{-Phe})$; $R_1 = R_3 = H$; R_2 = Phe.

Independently, Chládek *et al.* (1973) chemically synthesized the same peptidyl transferase acceptor, $Ado^{ox\text{-}red}$(Phe), and succeeded in the separation of both isomers. Their assays of peptidyl transferase activity confirmed that the 3′ isomer (IVa) was active and that the 2′ isomer (IVb) was inactive.

In order to show by direct methods that the product of enzymatic acylation of $tRNA^{ox\text{-}red}$ was the 2′ ester (IVb) we have now compared biosynthetic $Ado^{ox\text{-}red}$(Phe) with its chemically synthesized isomers by chromatography and by proton magnetic resonance spectroscopy. The results presented below show conclusively that essentially only the 2′ isomer is formed. This result is not a peculiarity of yeast Phe-tRNA synthetase since the same result was found when *E. coli* and rat liver enzymes acylate their homologous tRNAs, when rat liver enzyme acylates yeast $tRNA^{Phe}$, when *E. coli* enzyme acylates yeast $tRNA^{Phe}$ in the presence of 1.5 M ammonium sulfate, when yeast enzyme acylates *E. coli* $tRNA^{Phe}$ in the presence of 20% Me_2SO, and when yeast enzyme mischarges *E. coli* $tRNA^{Val}$ with phenylalanine in the presence of 20% Me_2SO.

Experimental Section

Oxidized-Reduced tRNAs. All $tRNA^{ox\text{-}red}$ preparations were made according to Ofengand and Chen (1972) except that a fivefold excess of rhamnose was used to stop the oxidation reaction, and the pH of the reducing solution (5 mg/ml of $NaBH_4$ in 0.2 M $K\text{-}PO_4$ (pH 7.8)) was adjusted to pH 7.5 by the addition of 2 μl of glacial acetic acid/ml of solution.

Acylation of tRNAs. Several different conditions for acylation with phenylalanine were used in this work. The standard assay, used for yeast synthetase with yeast or *E. coli* $tRNA^{Phe}$, was as described by Ofengand and Chen (1972) modified by the addition of 1 mM dithiothreitol and 10 μM each of the 19 other ^{12}C amino acids minus phenylalanine. The pH 5.8 cacodylate assay was as described by Roe *et al.* (1973) for mischarging of tRNAs, modified to contain 1 mM dithiothreitol and 20 μM [^{14}C]phenylalanine plus the 19 other ^{12}C amino acids. The Me_2SO-high Mg^{2+} assay conditions were the "special-high Mg^{2+}" conditions of Kern *et al.*, (1972) modified to contain 20 μM [^{14}C]phenylalanine plus the 19 other ^{12}C amino acids.

Rat liver synthetase acylation of rat liver and yeast tRNA with phenylalanine was performed as described by Tscherne *et al.* (1973) except that Bicine was substituted for Tris and 1 μM [^{14}C]phenylalanine plus the other 19 ^{12}C amino acids were used.

E. coli synthetase catalyzed acylation of *E. coli* $tRNA^{Phe}$ was performed in a mixture containing 100 mM Bicine (pH 7.5), 10 mM KCl, 10 mM $Mg(OAc)_2$, 2 mM ATP, 1 mM dithiothreitol, 10 μM [^{14}C]phenylalanine, plus the other 19 ^{12}C amino acids, synthetase, and tRNA. Incubation was at 37° and the reactions were stopped and assayed as previously described (Ofengand and Chen, 1972). For the *E. coli* synthetase catalyzed acylation of yeast $tRNA^{Phe}$ with phenylalanine, a modification of the ammonium sulfate conditions of Thiebe and Zachau (1970) was used. The reaction mixtures contained 10 mM Bicine (pH 7.5), 20 mM $Mg(OAc)_2$, 10 mM ATP, 1 mM dithiothreitol, 10 μM [^{14}C]phenylalanine plus the other 19 ^{12}C amino acids, 1.5 M $(NH_4)_2SO_4$, tRNA, and enzyme. Incubation was at 30°, and samples were analyzed as described above.

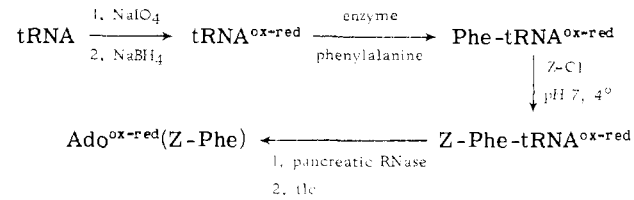

FIGURE 2: Flow sheet for analysis of acylation of oxidized-reduced tRNA.

Methionine acceptance assays of *E. coli* $tRNA_m^{Met}$ and valine acceptance assays of *E. coli* $tRNA^{Val}$ were performed as described previously (Krauskopf and Ofengand, 1971; Siddiqui and Ofengand, 1971). The mischarging of *E. coli* $tRNA^{Val}$ with phenylalanine by yeast synthetase was carried out exactly as described for the Me_2SO-high Mg^{2+} assay.

For preparative acylation, the above systems were used with time, enzyme concentration, and tRNA concentration adjusted to give maximal charging. Phe-$tRNA^{ox\text{-}red}$ was isolated from the charging mixture as previously described (Ofengand and Chén, 1972).

Large-Scale Preparation of $Ado^{ox\text{-}red}$(Z-Phe) from Yeast Phe-$tRNA^{ox\text{-}red}$. The general method is illustrated in Figure 2. $tRNA^{ox\text{-}red}$ was prepared from yeast phenylalanine tRNA and then enzymatically acylated with phenylalanine by yeast synthetase. Phe-$tRNA^{ox\text{-}red}$ was isolated as previously described (Ofengand and Chen, 1972). 65% recovery of charging activity was achieved in this preparation, with >93% of the Phe-tRNA being in the oxidized-reduced form as judged by tlc chromatography of the RNase degradation product (Ofengand and Chen, 1972).

The Phe-$tRNA^{ox\text{-}red}$ was then treated with *N*-benzyloxycarbonyl chloride (Z-Cl) (Stern and Littauer, 1968) to derivatize the free amino group of the phenylalanyl residue; 25 ml of Phe-tRNA (5.26 μM) was mixed with 25 ml of 1.0 M $K\text{-}PO_4$ (pH 7.0) at 4°, 2.5 ml of Z-Cl was immediately added, and the mixture was shaken vigorously for 10 min at 4°. Z-Cl (25 ml) was added at 10 min and at 20 min. At 30 min, the reaction was stopped by extraction with 100 ml of ethyl ether, and the extraction was repeated once. This procedure results in 65–85% derivatization. The aqueous phase was then dialyzed briefly against 0.05% KOAc, pH 5, at 4° and precipitated with 0.1 volume of 20% KOAc, pH 5, and 2.5 volume of ethanol at 0°. The precipitate was redissolved in 5 ml of 2% KOAc, pH 5, and reprecipitated. The resulting precipitate was still heavily contaminated by salts which were removed by repeated fractional precipitation. Of the Cl_3CCOOH precipitable radioactivity in the original dialyzed solution, 60% was eventually recovered. In later experiments (see below) the aqueous phase after ether extrac-

TABLE I: R_F Values of Phenylalanyladenosine Derivatives.[a]

Compound	Solvent A		Solvent B	
	Thick	Thin	Thick	Thin
Ado$^{ox\text{-}red}$(2′-Z-Phe)	0.28	0.75	0.15	0.42
Ado$^{ox\text{-}red}$(3′-Z-Phe)	0.34		0.15	0.48
Ado(Z-Phe)	0.52	0.89	0.28	
Ado$^{ox\text{-}red}$(2′-Phe)	0.01	0.14		
Ado(Phe)	0.03	0.26		
Z-Phe	0.66	0.93	0.10	0.06
Phe	0.04	0.18	0.01	0.01

[a] Solvent A, $CHCl_3$–MeOH–glacial HOAc (85:10:5 v/v); solvent B, $CHCl_3$–MeOH (90:10 v/v). Thick plates were 2 mm thick Merck PF 254 silica gel prepared in the Hoffmann-La Roche TLC Laboratory; thin plates were 100-μ fluorescent silica gel plastic-backed plates from Eastman (No. 6060). Compounds were prepared by treatment of the appropriate tRNA derivative with either alkali or pancreatic RNase, and detected by radioautography. The two isomers of Ado$^{ox\text{-}red}$(Z-Phe) were synthetic products (Chládek *et al.*, 1973) and were visualized under uv light.

TABLE II: Nmr Chemical Shift Data of the $H_{1'}$ Proton for Synthetic and Biosynthetic *N*-Benzyloxycarbonylphenylalanyl adenosine$^{ox\text{-}red}$.

Compound	$H_{1'}$ Observed Chemical Shift ppm (Me_4Si)
Synthetic Ado$^{ox\text{-}red}$(2′-Z-Phe) [I]	−6.345[a] (−6.33)[c]
Synthetic Ado$^{ox\text{-}red}$(3′-Z-Phe) [II]	−6.117[a] (−6.12)[c]
Synthetic MeOTr-Ado$^{ox\text{-}red}$ [III]	−6.095[a] (−6.10)[c]
Ado$^{ox\text{-}red}$(Z-Phe) isolated from tRNA	−6.339[b]

[a] Nmr spectra were obtained using a Varian HR 220 spectrometer equipped with a Fourier transform accessory and operating in the Fourier transform mode. The spectra were recorded at 22° using a 35-μsec pulse width and a 10-sec delay time between pulses. The solvent was acetone-d_6, 1% Me_4Si (Stohler Isotope Chemicals), and 10% D_2O v/v. The spectral resolution, determined by the full width of the Me_4Si resonance at half-height, was 1 Hz. The chemical shifts are reported in parts per million (ppm) downfield from Me_4Si. The synthetic compounds I–III were measured at concentrations of 1–10 mM in 150-μl volumes. [b] The biosynthetic isolate was analyzed as described in Figure 3. [c] Values from Chládek *et al.* (1973).

tion was desalted on Sephadex G-50 without resource to precipitation.

For RNase digestion, the mixture contained in 10 ml, 10.5 μM Z-Phe-tRNA$^{ox\text{-}red}$, 50 mM triethylammonium bicarbonate (pH 7.9), and 2 mg of pancreatic RNase (Worthington). This mixture (final pH 6.5) was incubated 15 min at 30°. The pH was brought to 5.0 with formic acid and lyophilized to 0.6 ml. Acetone (5.4 ml) was added, and the mixture was chilled to 0° for 15 min to precipitate salts and oligonucleotides; 90% of the radioactivity was recovered in the supernatant, which was concentrated in an air stream and then lyophilized.

The extract was then purified by tlc on thick plates in solvent A (Table I) to remove unidentified compounds with low R_F, any hydrolyzed Z-Phe, and, most importantly, any Ado(Z-Phe) which might have been formed either by the combined action of nuclease and CCA pyrophosphorylase contaminants during the acylation of tRNA with phenylalanine or by failure to completely oxidize the tRNA initially. In some preparations, a small amount of this contaminant was detected. The bands were located by radioautography and the silica gel was extracted with 90% acetone–water to recover the purified Ado$^{ox\text{-}red}$(Z-Phe); 40% of the radioactivity in the RNase digest was recovered in this fraction. After lyophilization to dryness, the sample was reextracted into 300 μl of 90% acetone to remove insoluble residues from the silica gel. Chromatography in solvent A (thick plate) and solvent B (thin plate) showed that less than 1% hydrolysis of the Ado(Z-Phe) had occurred. This sample was used both for the nuclear magnetic resonance analysis (Table II) and for the tlc analysis (Figure 4A).

Small-Scale Preparation of Ado$^{ox\text{-}red}$(Z-Phe) for Tlc Analysis. The same general method described above was also followed for all other isolations of Ado$^{ox\text{-}red}$(Z-Phe) from the various Phe-tRNA$^{ox\text{-}red}$ preparations. Oxidized-reduced tRNA was prepared, acylated, and isolated as described above. Reaction with Z-Cl was performed at 4° in a 1.0-ml reaction mixture containing 0.5 M K-PO_4 (pH 7.0), 0.07 M KOAc (pH 5), and 20–600 pmol of the Phe-tRNA. Z-Cl (50 μl) was added at 0 time, 10 min, and 20 min with continuous mixing on a Vortex mixer. Reaction was stopped at 30 min by extraction with 2 × 1 ml of ethyl ether, and the aqueous phase was desalted by gel filtration on Sephadex G-50 equilibrated in 0.05% KOAc (pH 5). The Z-Phe-tRNA peak was pooled and concentrated to 0.5 ml by lyophilization. Recovery of Cl_3CCOOH precipitable radioactivity was usually 85–90%.

RNase digestion was performed in 0.5 ml of 50 mM triethylammonium bicarbonate (pH 7.9) containing 6–400 pmol of Z-Phe-tRNA and 125 μg of pancreatic RNase. After 5 min at 30°, the mixture was lyophilized to 0.2 ml, 1.8 ml of acetone was added, and the mixture was kept at 0° for 5–10 min. After removal of the precipitate, the acetone supernatant containing at least 90% of the radioactivity in the digest was concentrated in a stream of air, and then analyzed by tlc as described below.

Tlc Analysis of the Amount of I and II Present. Two methods were used to quantitate the relative amount of I and II present after enzymatic acylation and derivatization. A mixture of synthetic I and II (Figure 1) was first added to the radioactive Ado$^{ox\text{-}red}$(Z-Phe) solution, and then the sample was analyzed by tlc. In method A, the sample was first chromatographed in solvent A (thick plate) to remove impurities as described above and to achieve an initial separation of the two isomers. The entire zone corresponding to each of the isomers, located by the position of the internal marker, was scraped from the plate and eluted serially with 90% acetone–water or 100% acetone. The first two elutions (2–3 ml each) sufficed to remove 90% of the radioactivity eluted by four elutions, which was the standard number used. The total cpm eluted from each zone was denoted I_i (or II_i). Further purification of each zone was carried out by reapplication of the sample, together with additional internal markers, to a thin silica gel sheet followed by elution with solvent B. After drying, the sheet was examined under uv light to locate the position of the I and II markers, cut into sections, and counted under 10 ml of toluene scintillation fluid. Typical results are illustrated in Figures 4 and 6.

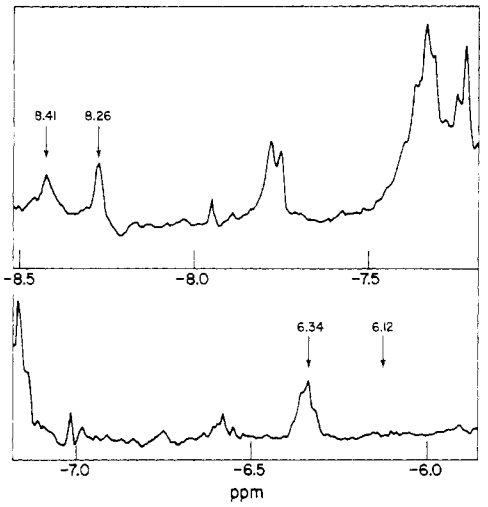

FIGURE 3: The 270-MHz proton nmr spectrum of Ado$^{ox\text{-}red}$(Z-Phe) isolated from tRNA. The spectrum was taken on a Bruker HX-270 spectrometer operating in the Fourier transform mode. The instrument was locked on the deuterium signal from the D_2O in the solvent. The spectrum was obtained at 14° using a 14-μsec pulse width for a 90° pulse and a 10-sec delay time between pulses. The solvent was acetone-d_6, 1% Me_4Si (Stohler Isotope Chemicals), and 10% D_2O v/v. The sample concentration was 0.30 mM in a volume of 100 μl. The integrated intensity of the $H_{1'}$ proton resonance was comparable to the integrated intensity of either the H_8 or H_2 proton resonance of the adenine ring which are the two lowest field resonances in this spectrum. The scale for the spectrum is in parts per million (ppm) relative to the frequency required for the Me_4Si resonance. The signal at −6.34 ppm is a triplet with separation of 5 Hz between peaks.

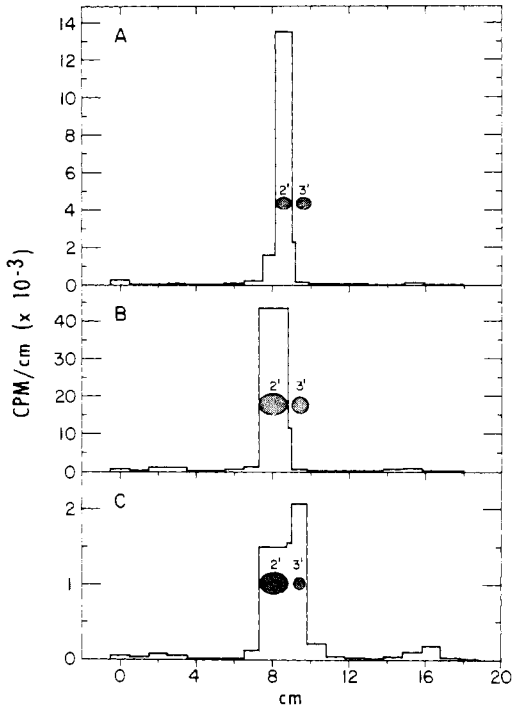

FIGURE 4: Tlc chromatography of Ado$^{ox\text{-}red}$(Z-Phe) isolated from different charged tRNAs. Each Ado$^{ox\text{-}red}$(Z-Phe) sample was prepared and purified as described in the Experimental Section. The tlc analysis was performed by method A. The shaded ovals show the positions of the marker compounds, synthetic I and II. Panel A, yeast synthetase was used to acylate yeast tRNA$^{ox\text{-}red}$ under standard conditions; panels B and C, yeast synthetase was used to acylate *E. coli* tRNA$^{ox\text{-}red}$ under Me_2SO–high Mg^{2+} conditions. Zones corresponding to I and II were eluted separately after tlc purification in solvent A, and analyzed in solvent B. Panel B; I; Panel C, II.

The fraction of the cpm in I_i (or II_i) which were truly I (or II) was obtained from this second chromatogram as the cpm at the I (or II) position divided by the sum of the cpm from all the sections of the strip. This fraction, f_I (or f_{II}) multiplied by I_i (or II_i), gave the true cpm in I (or II). The per cent II was then calculated as 100 times the true cpm in II divided by the sum of the true cpm in I plus the true cpm in II.

In method B, the sample was run in solvent A on a thick plate in two dimensions, two times in the first dimension, and three times in the same solvent in the second dimension. This sufficed to separate cleanly the I and II markers as well as all other contaminants. Radioautography confirmed the location of the radioactive spots. The entire zone of silica gel corresponding to the markers I and II was carefully scraped into scintillation vials and counted under 10 ml of toluene scintillation fluid. The per cent of II present was calculated as 100 times the radioactivity in II divided by that in the zones corresponding to I plus II.

In the case of yeast synthetase charging yeast tRNA, and rat liver synthetase charging yeast and rat liver tRNA, method A was modified by eluting both I and II together, before chromatography in solvent B. In this case, the per cent II was computed as for method B above.

tRNAs and Synthetases. Yeast tRNA^{Phe} (1030 pmol/A_{260}) and *E. coli* tRNA^{Val} (1082 pmol/A_{260}) were purchased from Boehringer, *E. coli* tRNA^{Phe} (1012 pmol/A_{260}) was a gift of Dr. B. S. Dudock, and *E. coli* tRNA$_m^{Met}$ (351 pmol/A_{260}, free of tRNA^{fMet}) was prepared as previously described (Henes *et al.*, 1969). Crude rat liver tRNA was donated by Dr. D. Grunberger. A mixed *E. coli* synthetase preparation was used as a source of Phe-, Val-, and Met-tRNA synthetases (Muench and Berg, 1966); the yeast mixed synthetase was prepared as described previously (Ofengand and Chen, 1972) or was a partially purified preparation (Roe *et al.*, 1973) donated by Dr. B. S. Dudock. The rat liver synthetase was purified according to Tscherne *et al.* (1973) and was the gift of Dr. J. Tscherne.

Other Materials. Synthetic compounds I, II, and III were prepared as described previously (Chládek *et al.*, 1973). All reagents not specifically mentioned were of the highest purity commercially available.

Results

Nmr Analysis of Yeast Phe-tRNA$^{ox\text{-}red}$. The initial motivation for this work was to show by direct methods that the product of enzymatic acylation of tRNA$^{ox\text{-}red}$ was the 2′-aminoacyl ester of tRNA$^{ox\text{-}red}$ (structure IVb of Figure 1 at the 3′ end). In order to do this recourse was had to nmr spectroscopy since it was known from previous work (Griffin *et al.*, 1966; Fromageot *et al.*, 1966; Sonnenbichler *et al.*, 1965) that the anomeric proton ($H_{1'}$) of 2′ derivatives of adenosine are more deshielded (and thus their resonance occurs at lower field) than are those of 3′ derivatives which hardly differ from unesterified nucleoside. This is also true for the open-chain analogs (Chládek *et al.*, 1973) (Table II). The *N*-benzyloxycarbonyl derivative of Phe-tRNA$^{ox\text{-}red}$ was used since (a) blockage of the amino group of phenylalanine decreased its rate of hydrolysis during the work-up, (b) Ado$^{ox\text{-}red}$(Z-Phe) was readily soluble in solvents such as acetone which greatly simplified both its isolation and the nmr measurements, and (c) it allowed an exact comparison with synthetic compounds previously characterized (Chládek *et al.*, 1973). Derivatization of Phe-tRNA$^{ox\text{-}red}$ was carried out essentially as described by

TABLE III: Acylating Capacity of Oxidized–Reduced and Control tRNAs in Homologous and Heterologous Combination.[a]

		Specific Activity (pmol/A_{260})							
		$tRNA^{Phe}_{yeast}$		$tRNA^{Phe}_{coli}$		$tRNA^{mixed}_{rat}$		$tRNA^{Val}_{coli}$	
Enzyme	Assay Condition	Control	Ox–red	Control	Ox–red	Control	Ox–red	Control	Ox–red
E. coli	Standard	117	2	1012	400			1082	282
	1.5 M $(NH_4)_2SO_4$	739	508	1003	713				
Yeast	Standard	740	690	40	3				
	pH 5.8 cacodylate	728	665	365	50				
	Me_2SO–high Mg^{2+}	637	586	496	507				88
Rat liver	Standard		236			38	30		

[a] Assay conditions were as described in the Experimental Section. Some of the data are summarized from Table IV and Figure 5, using the average maximum values reached. The remaining specific activities were obtained either from the plateau value of a time course experiment at a fixed tRNA concentration or from a tRNA linearity curve.

Stern and Littauer (1968), and after RNase treatment, the resulting Ado(Z-Phe) ester was purified by tlc chromatography. Nmr analysis of the H_1' resonance in the final product is shown in Table II and Figure 3. The values for the synthetic standards agree very well with the previously published data, and it is immediately clear that the biosynthetic isolate corresponds to the 2′ isomer. No peak corresponding to the 3′ isomer was detected, but less than 15% might have escaped detection (Figure 3).

Chromatographic Analysis of Yeast Phe-tRNA$^{ox\text{-}red}$. Since the above method, while a direct physical approach, was limited in terms of sensitivity, recourse was had to tlc chromatography or radioactively labeled material. Chládek *et al.* (1973) had already shown that it was possible to distinguish chromatographically between the 2′ and 3′ esters of Ado$^{ox\text{-}red}$(Z-Phe), structures I and II of Figure 1, and this made it possible to analyze in a simple way for the amount of II in the biosynthetic product by using radioactive phenylalanine in the acylation reaction. The separation of I and II typically found is illustrated in Figure 4A and summarized in Table I. The same preparation used for nmr analysis was examined, and Figure 4A shows that virtually no 3′ isomer was present, the actual amount being 0.75% (Table V, line 1). Clearly, acylation of yeast $tRNA^{Phe}$ by yeast synthetase occurs almost exclusively on the 2′-hydroxyl, in direct confirmation of our previous conclusions.

Analysis of Other Acylation Systems. The availability of a simple chromatographic assay made it possible to extend these observations to other tRNA synthetase systems in order to test the generality of the phenomenon. Previous work (Hussain and Ofengand, 1973; Sprinzl and Cramer, 1973) had been confined to the yeast $tRNA^{Phe}$–yeast synthetase system. In order to take advantage of the known properties and availability of synthetic marker compounds, our studies were limited to the charging of phenylalanine onto tRNA. Within this limit, however, we have examined three homologous systems (yeast synthetase–yeast $tRNA^{Phe}$, *E. coli* synthetase–*E. coli* $tRNA^{Phe}$, rat liver synthetase–rat liver $tRNA^{Phe}$), three heterologous systems (yeast synthetase–*E. coli* $tRNA^{Phe}$, *E. coli* synthetase–yeast $tRNA^{Phe}$, rat liver synthetase–yeast $tRNA^{Phe}$), and one heterologous mischarging system (yeast synthetase–*E. coli* $tRNA^{Val}$). The extent of charging obtained in these different systems is summarized in Table III and further discussed below.

Acylation with Yeast Synthetase. The kinetics of acylation of yeast and *E. coli* $tRNA^{ox\text{-}red}$ with phenylalanine catalyzed by yeast synthetase under different conditions is shown in Figure 5. Panel A confirms (a) that oxidized–reduced yeast $tRNA^{Phe}$ can be acylated almost as well as the control (Cramer *et al.*, 1968; Chen and Ofengand, 1970) and (b) that *E. coli* $tRNA^{Phe}$ cannot be acylated by yeast synthetase under standard conditions (Thiebe and Zachau, 1968). Panel B shows that the special assay conditions developed by Dudock (Roe and Dudock, 1972; Roe *et al.*, 1973) for charging *E. coli* $tRNA^{Phe}$ are effective with the control $tRNA^{Phe}$ but do not allow acylation of oxidized–reduced *E. coli* $tRNA^{Phe}$. Acylation to about 40% of the homologous activity is in agreement with our previous experience with these heterologous conditions (Kumar *et al.*, 1973) although Roe *et al.* (1973) have reported a greater degree of acylation. However, panel C shows that if conditions are used that lead to even a greater relaxation of the specificity between tRNA and synthetase (Kern *et al.*, 1972) namely 20% Me_2SO and 40 mM Mg^{2+}, the oxidized–reduced *E. coli* $tRNA^{Phe}$ could be acylated to the same extent as the control. As shown in the figure and summarized in Table III, both sets of special assay conditions did not materially affect the degree of acylation of either control or oxidized–reduced yeast $tRNA^{Phe}$, when acylation was compared at the optimal times.

The conditions of Figure 5C were used to prepare *E. coli* Phe-$tRNA^{ox\text{-}red}$ with yeast synthetase, and after reaction with Z-Cl and isolation of the Ado$^{ox\text{-}red}$(Z-Phe), it was analyzed by chromatography. The result is shown in Figure 4B

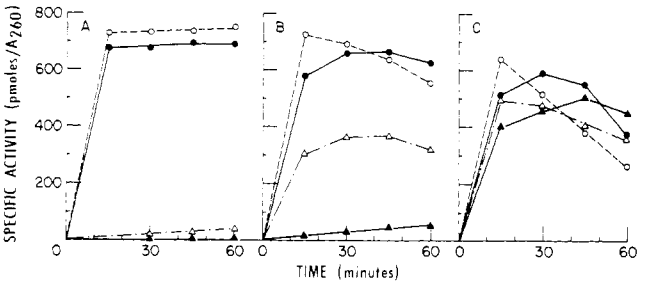

FIGURE 5: Time course of acylation of yeast and *E. coli* $tRNA^{ox\text{-}red}$ with yeast synthetase under different conditions. Untreated control and oxidized–reduced $tRNA^{Phe}$ of yeast and *E. coli* were acylated with phenylalanine by yeast synthetase under (a) standard, (b) pH 5.8 cacodylate, and (c) Me_2SO–high Mg^{2+} acylation conditions as described in the Experimental Section. Untreated yeast tRNA, ○; yeast $tRNA^{ox\text{-}red}$, ●; untreated *E. coli* tRNA, △; *E. coli* $tRNA^{ox\text{-}red}$, ▲.

TABLE IV: Acylation of Yeast and *E. coli* $tRNA^{ox\text{-}red}$ with Phenylalanine in the Presence of 1.5 M Ammonium Sulfate.

Incubation Time (min)	1.5 M $(NH_4)_2SO_4$	Specific Activity (pmol/A_{260}) $tRNA^{Phe}_{yeast}$ Control	$tRNA^{Phe}_{yeast}$ Ox-red	$tRNA^{Phe}_{coli}$ Control	$tRNA^{Phe}_{coli}$ Ox-red
30	–	65	<1	959	366
45	–	86	<1	1069	
60	–	117	2	1009	432
30	+	786	440	1090	743
45	+	692	490	915	683
60	+		526	809	542

[a] Assays were performed as described in the Experimental Section for acylation of yeast $tRNA^{Phe}$ with *E. coli* synthetase in the presence of 1.5 M ammonium sulfate.

and C, and the analysis is summarized in Table V. Even under these unusual conditions, the fidelity of acylation at the 2′ position is preserved.

Next, an even more abnormal case was examined, that of the mischarging of *E. coli* $tRNA^{Val}$ with phenylalanine catalyzed by yeast synthetase. This reaction has been extensively studied in Dudock's laboratory (Roe *et al.*, 1973) and by Ebel and coworkers (Kern *et al.*, 1972) using the pH 5.8 cacodylate and Me_2SO–high Mg^{2+} conditions, respectively. Since oxidized–reduced *E. coli* $tRNA^{Phe}$ was not charged in the pH 5.8 cacodylate system, only the Me_2SO–high-Mg^{2+} conditions were tried for acylation of oxidized–reduced $tRNA^{Val}$. As shown in Table III, it was possible to acylate this tRNA to 31% of the level reached in the homologous assay which used *E. coli* synthetase and valine. The $Ado^{ox\text{-}red}$(Z-Phe) derived from this tRNA was analyzed by method B, and the results are summarized in Table V.

Acylation with E. coli Synthetase. It was first shown by Thiebe and Zachau (1970) that *E. coli* synthetase could be induced to acylate yeast $tRNA^{Phe}$ with phenylalanine by including 1.5 M $(NH_4)_2SO_4$ in the reaction. This finding was confirmed by the experiment shown in Table IV, which also shows that oxidized–reduced tRNAs have the same properties, and that 1.5 M $(NH_4)_2SO_4$ does not affect homologous charging. In the absence of ammonium sulfate, oxidized–reduced yeast $tRNA^{Phe}$ could not be acylated, but in its presence it was acylated almost as well as control yeast $tRNA^{Phe}$ or oxidized–reduced $tRNA^{Phe}$ assayed with yeast enzyme under standard conditions (see Table III). It is interesting to note that ammonium sulfate also increased the maximum degree of acylation of *E. coli* oxidized–reduced $tRNA^{Phe}$, suggesting that a less than maximal plateau of charging in oxidized–reduced tRNAs (Ofengand and Chen, 1972; Tal *et al.*, 1972) may in some cases only be due to the assay conditions used.

Preparative-scale charging and isolation of Ado(Z-Phe) derived from *E. coli* $tRNA^{Phe}$ and yeast $tRNA^{Phe}$ acylated by *E. coli* enzyme under standard and ammonium sulfate conditions, respectively, were carried out and the products subjected to tlc analysis. In this series, analysis was carried out both by method A and by method B. The result of the method A analysis is shown in Figure 6 and summarized in Table V, while the method B analysis (two-dimensional chromatography) is not shown, but the results are given in Table V. There is good agreement between both methods of analysis.

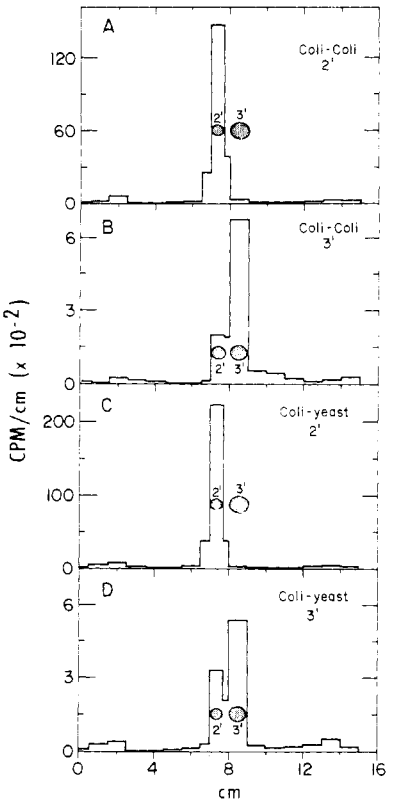

FIGURE 6: Tlc chromatography of $Ado^{ox\text{-}red}$(Z-Phe) isolated from yeast and *E. coli* tRNA acylated with *E. coli* enzyme. Each sample was prepared, purified, and analyzed by method A as described in the Experimental Section. The shaded ovals show the location of markers I and II. Panels A and B, *E. coli* synthetase was used to acylate *E. coli* $tRNA^{ox\text{-}red}$ under standard conditions. Zones corresponding to I and II were eluted and analyzed separately. Panel A, Zone I; panel B, Zone II. Panels C and D: *E. coli* synthetase was used to acylate yeast $tRNA^{ox\text{-}red}$ in the presence of 1.5 M ammonium sulfate. Zones I and II were eluted and analyzed separately. Panel C, zone I; panel D, zone II.

Acylation with Rat Liver Synthetase. Since rat liver synthetase readily acylates yeast tRNA in addition to its cognate tRNA (Tscherne *et al.*, 1973) both of these oxidized–reduced tRNAs were examined. As shown in Table III, crude rat liver $tRNA^{ox\text{-}red}$ could be acylated almost as well as the control, but yeast $tRNA^{ox\text{-}red}$ was acylated to a lower degree (compare 236 pmol/A_{260} for rat enzyme to 690 pmol/A_{260} for yeast enzyme).

Both tRNAs were charged and derivatized on a preparative scale, and their respective Ado(Z-Phe) derivatives were isolated and analyzed by tlc chromatography by method A (Table V).

Discussion

As pointed out in the introduction, earlier work (Hussain and Ofengand, 1973) had shown that acylation of oxidized–reduced $tRNA^{Phe}$ must have occurred on the 2′-hydroxyl, but the evidence, while clear, was indirect. In the meantime, Sprinzl and Cramer (1973) showed that a modified yeast $tRNA^{Phe}$ with either 2′- or 3′-deoxyadenosine at the 3′ end could be prepared and that tRNA-C-C-3′-dA could be acylated, but tRNA-C-C-2′-dA could not. The evidence presented here shows that by nmr spectroscopy more than 85% and by tlc chromatography greater than 99% of yeast phenylalanine $tRNA^{ox\text{-}red}$ is acylated at the 2′ position. Could isomerization during the work-up and analysis have produced this result? Previous work (Hussain and Ofengand, 1973; Chládek *et al.*, 1973) did not show any indication of

TABLE V: Per Cent Acylation at 3′-Hydroxyl by Various Synthetase–tRNA Combinations.

tRNA	Per Cent Acylation at 3′-Hydroxyl: Yeast Synthetase	*E. coli* Synthetase	Rat Liver Synthetase
Yeast $tRNA^{Phe}$	*0.75*	3.3, 2.5* [1.5 M $(NH_4)_2SO_4$]	7
E. coli $tRNA^{Phe}$	2.5 [20% Me_2SO]	*4.4, 4.4**	
E. coli $tRNA^{Val}$	2.0* [20% Me_2SO]		
Rat liver $tRNA^{Phe}$			*9*

[a] The data are summarized from Figures 4 and 6 and additional experiments. The analysis of rat liver synthetase acylations was made by method A as described in the Experimental Section with both I and II being eluted together after tlc purification. The starred (*) analyses were made by method B. Homologous combinations of synthetase and tRNA are italicized.

isomerization in the open-chain analogs, and the present results also show that this does not occur. Chromatography of synthetic I and II has never shown any conversion of one compound to the other, and the finding of from 1 to 10% of the 3′ isomer II in different acylation reactions after an identical work-up also rules out the possibility of isomerization occurring at an earlier stage.

The two methods of tlc analysis used in this work were directly compared in two cases (Table V) and both were shown to give equivalent results. The main purpose of chromatography in solvent A on thick plates was to remove any contaminating Ado(Z-Phe) and this was done equally well by method B.

The final results, summarized in Table V, show clearly that different synthetases acylate the 3′-hydroxyl to different extents, which are relatively independent of the nature of the acceptor tRNA and of the reaction conditions employed. It was fortuitous that yeast synthetase was the enzyme examined in earlier work, since that enzyme gives the most clear-cut results. However, the finding that yeast, *E. coli,* and rat liver synthetases can acylate the 3′-hydroxyl to a limited extent should not be allowed to obscure the fact that in all systems studied, including heterologous and mischarging reactions under extremely abnormal conditions, the predominant product was the 2′ isomer. This result indicates to us that acylation of the 2′-hydroxyl of normal tRNA is the natural mechanism of action of aminoacyl-tRNA synthetases. Contrary arguments which propose that the 2′-hydroxyl of oxidized–reduced tRNA is fortuitously oriented like the 3′-hydroxyl of normal tRNA become difficult to accept in view of the preservation of isomer specificity even in 1.5 M ammonium sulfate and 20% dimethyl sulfoxide. Indeed, what ability there is to acylate the 3′-hydroxyl appears to be more a property of the synthetase than of the tRNA. The fact that a structurally different analog of tRNA, tRNA-C-C-dA, also gives the same results (Sprinzl and Cramer, 1973) adds further force to our argument.

The biological reason for the observed specificity of acylation is not known. Were aminoacyl-tRNA ever free in the cytosol of the cell, the amino acid would equilibrate between the 2′ and 3′ positions almost instantly (McLaughlin and Ingram, 1965; Griffin *et al.,* 1966) so that any functional reason for the enzymatic specificity of acylation would disappear. If, however, aminoacyl-tRNA normally is handed on from synthetase to elongation factor without any appreciable lifetime free in solution, one may consider the consequences of isomer specificity. Elongation factor EFTu-dependent binding to the ribosomal A site of the deoxy-tRNA analog, tRNA-C-C-3′-dA(2′Phe), has been demonstrated (Chinali *et al.,* 1974) so that isomerization to the 3′ ester is not required at this step. The only other nonisomerizable tRNA analog so far studied, $Phe\text{-}tRNA^{ox\text{-}red}$, does not bind to EFTu-GTP (Ofengand and Chen, 1972). Function at the A site as an acceptor, however, appears to be completely (Hussain and Ofengand, 1973; Chládek *et al.,* 1973; Ringer and Chládek, 1974; Chládek *et al.,* 1974) or at least largely (Hussain, 1974; Chinali *et al.,* 1974) specific for the 3′ isomer. Consequently, a ribosome-catalyzed isomerization from 2′ to 3′ has been proposed to occur at the A site of the ribosome (Ringer and Chládek, 1974; Chládek *et al.,* 1974), in order to account for these results. A similar migration from 2′ to 3′ during protein synthesis had been earlier hypothesized by Zamecnik (1962). There is as yet no direct evidence for such a reaction, however, nor is it yet clear what functional purpose it would serve. These topics clearly will require further research.

The general effect of oxidation–reduction at the acceptor end of tRNA, summarized in Table III, is to reduce the maximum level of acylation. This effect, also noted by Tal *et al.* (1972), may however, be more apparent than real. For example, the charging maximum of oxidized–reduced *E. coli* $tRNA^{Phe}$ was increased from 400 to 713 pmol/A_{260} simply by addition of ammonium sulfate to the acylation mixture (Table IV), and the specific activity of *E. coli* oxidized–reduced $tRNA_m^{Met}$ was increased threefold by assay in 20% Me_2SO–40 mM Mg^{2+}, conditions which had no effect on the charging of control $tRNA_m^{Met}$ (data not shown). It seems likely that the apparent maximum acylation observed is really a steady-state balance between acylation and deacylation of the type analyzed by Bonnet and Ebel (1972), and that oxidized–reduced tRNA is, in most cases, analogous to a heterologous or incorrect tRNA. Thus it may be generally possible to induce extensively modified tRNAs which show a reduced or zero acylation activity to accept amino acid by the use of special charging conditions.

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Affinity Labeling of *Escherichia coli* Ribosomal Proteins with an Analog of the Natural Initiator tRNA†

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ABSTRACT: Bromoacetylmethionyl-tRNA^{fMet} is an analog of the peptide chain initiator formylmethionyl-tRNA^{fMet}. Its binding to unwashed 70S *Escherichia coli* ribosomes is enhanced 18-fold in the presence of bacteriophage f2 RNA. f2 RNA does not promote the binding of the analog to salt-washed ribosomes. This presumably indicates that the process requires initiation factors. Addition of alanyl-tRNA, in the form of an alanyl-tRNA · EF-Tu · GTP complex, to the reaction mixture containing the analog bound to ribosomes in the presence of f2 RNA results in the attachment of about 0.6 mol of alanyl residues/mol of analog attached. This is taken as evidence for the formation of a substituted methionylalanyl dipeptide and is the expected result since alanine is the second amino acid in the coat protein translated from f2 RNA *in vitro*. About 5% of the labeled analog and of the labeled alanyl residues were found to be covalently linked to 50S proteins. One protein, L2, contained four times as much covalently attached label as any other 50S protein. In another experiment in which only the labeled analog was bound to ribosomes L2 again was the most heavily labeled. A smaller amount of label was attached to L27. In the absence of f2 RNA or 30S subunits only 5% as much analog became linked to 50S proteins as in a complete system. These results are in line with earlier conclusions from experiments with poly(uridylic acid) and a phenylalanyl-tRNA analog. They both indicate that L2 and perhaps L27 may be part of the peptidyl-tRNA binding site (P site) of the 50S subunit or are at least close to this site.

The determination of the location within the ribosome and function in protein synthesis of particular ribosomal proteins is under way. One of the approaches to this problem is based on affinity labeling. This involves the use of certain analogs of substrates or inhibitors of reactions occurring on the ribosome. Such a substrate analog carries a chemically reactive group. Upon binding of the analog to the ribosome the reactive group is potentially able to form a covalent linkage between the analog and particular ribosomal components. Any ribosomal component which reacts with the analog can then be identified. If other explanations can be ruled out, it is assumed that such a ribosomal component is part of the binding site or is located close to the binding site of the substrate. The validity of this assumption is greatly supported if it can be shown that the substrate analog functions correctly after it has been linked covalently to the ribosome.

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